Exploring Molecular Mechanisms Controlling Skin Homeostasis and Hair Growth

MicroRNAs in Hair-cycle-Dependent Gene Regulation, Hair Growth and Associated Tissue Remodelling

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Dedicated

To my daughter Misba Ahmed and my Family

Abud-Darda (May Allah be pleased with him) reported;

The messenger of Allah (PBUH) said, "He who follows a path in quest of knowledge, Allah will make the path of Jannah (heaven) easy to him. The angels lower their wings over the seeker of knowledge, being pleased with what he does. The inhabitants of the heavens and the earth and even the fish in the depth of the oceans seek forgiveness for him. The superiority of the learned man over the devout worshipper is like the full moon to the rest of the stars (i.e., in brightness). The learned are the heirs of the Prophets who bequeath neither dinar nor dirham (i.e., money) but only that of knowledge; and he acquires it, has in fact acquired an abundant portion."

Narrated by Abu Dawud and At-Tirmidhi (600AD)

..The key to success is to hold your head up high, walk straight, never waiver in your beliefs and praise those who have helped you on your way. Success comes to those who follow their dreams with determination and who take pride and risks on behalf of destiny. When you take up the challenge to help and inspire others and truly make a difference to their lives, therein lays true success and happiness.

"If I have seen further (than certain other men) it is by standing upon the shoulders of giants." Sir Isaac Newton

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Abstract

The hair follicle (HF) is a cyclic biological system that progresses through stages of growth, regression and quiescence, each being characterized by unique patterns of gene activation and silencing. MicroRNAs (miRNAs) are critically important for gene silencing and delineating their role in hair cycle may provide new insights into mechanisms of hair growth control and epithelial tissue remodelling.

The aims of this study were: 1) To define changes in the miRNA profiles in skin during hair cycle-associated tissue remodelling; 2) To determine the role of individual miRNAs in regulating gene expression programs that drive HF growth, involution and quiescence; 3) and to explore the role of miRNAs in mediating the effects of BMP signalling in the skin.

To address Aims 1 & 2, global miRNA expression profiling in the skin was performed and revealed marked changes in miRNAs expression during distinct stages of the murine hair cycle. Specifically, miR-31 markedly increased during anagen and decreased during catagen and telogen. Administration of antisense miR-31 inhibitor into mouse skin during the early- and mid-anagen phases of the hair cycle resulted in accelerated anagen development, and altered differentiation of hair matrix keratinocytes and hair shaft formation. Microarray, qRT-PCR and Western blot analyses revealed that miR-31 negatively regulates expression of Fgf10, the components of Wnt and BMP signalling pathways Sclerostin and BAMBI, and Dlx3 transcription factor, as well as selected keratin genes. Luciferase reporter assay revealed that Krt16, Krt17, Dlx3, and Fgf10 serve as direct miR-31 targets.

In addition, miR-214 was identified as a potent inhibitor of the Wnt signalling pathway in the keratinocytes. Mutually exclusive expression patterns of miR-214 and β-catenin was observed during HF morphogenesis. MiR-214 decreases the expression of β-catenin and other components of Wnt signalling pathways c-myc, cyclin D1, and Pten in the keratinocytes. Luciferase reporter assay proved that β-catenin serves as a direct target of miR-214. In addition, miR-214 prevented translocation of β-catenin into the nucleus in response to the treatment with an activator of the Wnt signalling pathway lithium chloride, and abrogated the lithium-induced increase of the expression of the Wnt target gene

Axin2. This suggests that miR-214 may indeed be involved in regulation of skin development and regeneration at least in part, by controlling the expression of β-catenin and the activity of the Wnt signalling pathway.

To address Aim 3, the role of miRNAs in mediating the effects of the bone morphogenetic protein (BMP) signalling in the skin was explored. MiRNAs were isolated from the primary mouse keratinocytes treated with BMP4 and processed for analysis of global miRNA expression using the microarray approach. Microarray and real-time PCR analysis revealed BMP4-dependent changes in the expression of distinct miRNAs, including miR-21, which expression was strongly decreased in the keratinocytes after BMP4 treatment. In contrast, miR-21 expression was substantially higher in the skin of transgenic mice over-expressing BMP antagonist Noggin. Transfection of the keratinocytes with miR-21 mimic revealed existence of two groups of the BMP target genes, which are differentially regulated by miR-21. Thus, this suggests a novel mechanism controlling the effects of BMP signalling in the keratinocytes.

Thus, miRNAs play important roles in regulating gene expression programs in the skin during hair cycle. By targeting a number of growth regulatory molecules, transcription factors and cytoskeletal proteins, miRNAs are involved in establishing an optimal balance of gene expression in the keratinocytes required for the HF and skin homeostasis.

Key words: MicroRNAs, Hair follicle, Growth control, Global miRNA expression, Keratin 16, Keratin 17, Dlx3, and Fgf10

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Abbreviations

Ago	Argonaute
APS	Ammonium Persulphate
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
BDNF	brain-derived neurotrophic factor
BMPs	Bone mophogenic proteins
BMPR1A	Bone mophogenic proteins receptor 1a
CDP	*Cutl1* gene encoding - CCAAT displacement protein
Ct	Threshold cycle
CTS	Connect tissue sheath
DAPI	4', 6-diamidino-2-phenylindole
DEPC	Diethyl-pyrocarbonate
DGRC8	DiGeorge Syndrome Critical Region Gene 8
DIG	Digoxigenin
Dkk	Dickkopf
DMBA	dimethylbenz[a]antracen
DMEM	Dulbecco’s modified Eagle’s medium
DP	Dermal Papilla
Dpd	Days of postnatal depilation
ECM	Extracellular Matrix
Eda	Ectodysplasin
EdaR	Ectodysplasin receptor
EGA	Estimated gestation age
EGF	Epidermal growth factor
EMEM	Eagle's minimum essential medium
Exp 5	Exportin 5
FAP	Familiar Adenomatous Polyposis
FGF	Fibroblast growth factor
FISH	Fluorescent In situ hybridization
FP	Follicular papilla
GFP	Green fluorescent protein
GTP	Guanosine triphosphate

GSK	Glycogen synthase kinase
HF	Hair follicle
HGF	Hepatocyte growth factor
hrr	Hairless gene
IAP	Inhibitor of Apoptosis
IGF	Insulin growth factor
IRAK	IL-1R-associated kinase
IRS	Inner root sheath
ISH	In situ hybridization
K	Keratin
KGF	keratinocyte growth factor
KIFS	Keratin intermediate filaments and keratin-associated proteins
Krts	Keratins
Lef	lymphoid enhancer factor
LNA	Locked Nucleic Acid
LRC	label retaining cells
LGR	Leucine-rich containing G-protein-coupled receptor
MC1R	Melanocortin 1 receptor
miR	MicroRNA
miRNAs	MicroRNA
MRE	MicroRNA recognition element
mRNA	Messenger RNA
MSH	Melanocyte stimulating hormone
NBT	4-Nitro blue tetrazolium cholride
ncRNA	Non-coding RNA
NT	Neurotrophin
ORS	Outer root sheath
PAZ	Piwi-Argonaute-Zwille
PBS	Phosphate saline buffered solution
PDGF-A	Platelet-derived growth factor-A
Pol II	RNA polymerase II
Pre-miRNA	Precursor microRNA

Pri-miRNA	Primary microRNA
PMEKs	Primary mouse epidermal keratinocytes
PTGS	Post-transcriptional gene silencing
RBP	RNA binding protein
RISC	RNA induced silencing complex
RNAi	RNA interference
rRNA	Ribosomal RNA
SCC	Squamous cell carcinoma
SCF	Stem cell factor
SDS	Sodium dodecyle sulphate
SHG	Secondary hair germ
Shh	Sonic hedgehog
siRNA	Small interfering RNA
Ptch	Patched
snRNA	Small nuclear RNA
SOCS-3	Cytokine signalling-3
STAT	Activator of transcription 3
TCF	T cell factor
TG	Transgenic
TGF	Transforming growth factor
TNF	Tumour necrosis factor
TPA	2-tetradecanoil-phorbol-13-acetate
TRAF	TNF receptor-associated factor
TRBP	The human immunodeficiency virus trans-activation response RNA-binding protein
tRNA	Transfer RNA
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
UTR	Untranslated region
VDR	Vitamin D receptor
VEGF	Vascular endothelial growth factor
Xiap	X linked inhibitor of apoptosis
4-OHT	4-hydroxytamoxifen

Chapter I

INTRODUCTION

1.0 Introduction

1.1Structure and Functions of the skin

The skin is the largest organ of the human body and it's most important function is to form a physical barrier against external environmental insults including, mechanical, chemical, and microbial (Proksch *et al.,* 2009; Jensen and Proksch, 2009). The skin further provides protection by regulating the loss of internal bodily fluids by controlling changes in body temperature. In addition, the skin is water resistant, and contains a advanced sensory network for pressure sensations and quick responses to hot and cold environments (Tobin and Kauser, 2005; Tobin, 2006; Fuchs 2007; Koster and Roop 2007; Brenner and Hearing, 2008; Fuchs and Horsely, 2008; Blanpain, 2010).

The skin can be divided into two major parts, the epidermis, and dermis. The epidermis and several appendages of the skin, represent the ectodermal compartment of the skin, while the dermis and hypodermis are of mesodermal origin (Holbrook and Wolff, 1993). Keratinocytes are the largest cell population in the skin and are responsible for the formation of terminally differentiated layers found within the epidermis. Keratinocytes produce keratins, which are the structural proteins that create the intermediate filament in epithelial cells (Moll *et al.*, 1982; Tobin, 2006).

1.1.1 Epidermis

The epidermis is divided into the following 5 sublayers or strata: basal, spinosum, granulosum, lucidum, and corneum. The basal layer is attached to the basement membrane, which separates the epidermis from the dermis. These basal keratinocytes are rapidly proliferating cells and represent the unique cell population of epidermal stem cells and transiently amplifying progenitors cells, which can give rise to terminally differentiated layers of the stratum spinosum (spinous layer), granular layer (stratum granulosum), and stratum corneum (horny layer) (Alonso and Fuchs, 2003). This differentiation program of basal keratinocytes constantly occurs throughout the life of humans and mammals and allows the replenishment of terminally differentiated cells, which have been sloughed off (Tobin, 2006; Alonso and Fuchs, 2003). It has been shown that the keratinocytes of the basal layer express specific keratins including; keratin 5 and keratin 14, respectively (Bowden *et al.*, 1987; Rao *et al.*, 1996). As basal cells begin to differentiate and enter the first suprabasal layer, a decrease in expression of keratin 5 and keratin 14 is observed (Rao *et al.*, 1996). In addition to the keratinocytes, the basal layer also contains other cell populations including Merkell, Langerhans cells, and melanocytes. Merkel cells are responsible for the transmission of tactile sensation through the skin's nerve (Moll *et al.*, 2005; Tobin, 2006). Langerhans cells are antigen presenting bone marrow-derived dentritic cells, which have a crucial role in immunological reactions. Melanocytes are specialized neural-crest-derived cells producing pigment (melanin), which provides coloration and protects humans against the damaging effects of UVR radiation (Tobin and Kauser, 2005; Koch *et al.*, 2006; Costin and Hearing, 2007).

The next level above the basal layer is the stratum spinosum (spinous layer), containing 8-10 sheets of keratinocytes. The spinous layer contains a web-like system of interfillaments attached to a specialized cell junction known as corneo-desmosomes, which help to resist physical trauma. The keratinocytes of spinous layer express keratin 1 and keratin 10 and these keratins are only expressed in differentiating epidermal keratinocyte cells (Bowden *et al.*, 1987; Rao *et al.*, 1996; Zhu *et al.,* 1999; Tobin, 2006). Keratinocyte differentiation in the epidermis also involves induction of proteins such as: involucrin, transglutaminase, fillagrin and loricrin, which forms the highly cross-linked layer- the cornified cell envelope. This process represents the final stage of epidermal keratinocyte differentiation (Fuchs and Green, 1980; Eckert and Rorke, 1989; Rao *et al.*, 1996).

The next, upper layer is the granular layer of the epidermis, a 3-5 sheets of non-proliferating keratinocytes producing two types of granules. The keratohyaline granules represent a mixture of smaller proteins containing keratohyaline and filaggrin (Matoltsy and Matoltsy, 1970). The lamellated granules are specialized secretory granules, which contain lipids and their extracellular processing enzymes.The granules increase in number and size and as the cell nuclei breakdown, their cell membrane increasingly becomes impermeable. These cells are then flattened as proliferating cells underneath them gradually push them toward the skin surface (Tobin, 2006; Costin and Hearing, 2007).

The stratum corneum is the outermost layer of the epidermis consisting of 15-30 sheets of keratinocytes, which are terminally differentiated (non-viable) but

biochemically-active cells called corneocytes. The corneocytes are connected by corneo-desmosomes and embedded in a lipid-rich matrix containing specialized proteins and lipids (Elias, 1983; Madison, 2003; Elias, 2005; Tobin, 2006). The final stages of keratinocyte differentiation are linked to dramatic changes in the structure of keratins, leading to the transformation of these cells into flat and anucleated corneocytes, surrounded by a cell envelope. Overtime, these cells slough off and are replaced by underlying differentiating keratinocytes (Madison, 2003; Proksch *et al.*, 2008).

1.1.2 Dermis

Immediately below the epidermis, is the dermis. The dermis can be divided into two distinct layers, the upper papillary and lower reticular regions. These layers are different in the arrangement of collagen fibrils, connective tissue components, cell number, and supply of blood vessels and nerves in the dermis (Cormack, 1987; Sorrell and Caplan, 2004; Tobin, 2006). The main cell type of the dermis is the fibroblast, a migratory cell that makes and degrades extracellular matrix components (Chang *et al.,* 2002). The superficial papillary layer of the dermis is arranged into ridge-like structures, containing micro-vascular and neural components that maintain the epidermis (Cormack, 1987; Sorrell and Caplan, 2004). The reticular layer of the dermis extends from the papillary layer to the deeper vascular plexus (Cormack, 1987; Sorrell and Caplan, 2004). The reticular layer makes up approximately 80% of the thickness of the dermis. The reticular layer ECM contains collagen and elastin fibers (Kurt, 1991). The collagen provides the skin with strength and tissue integrity whereas elastin provides

elasticity and resiliency (Kurt, 1991; Haake and Holbrook, 1999; Brucker-Tuderman, 2003; Costin and Hearing, 2007).

1.1.3 Skin appendages

Skin appendages such as nails, hair follicles, feathers, teeth and a number of glands including mammary glands are all derivatives of the embryonic ectoderm. Even though fully formed skin appendages diverge greatly in number, shape, function, and regenerative capacity, the early stages in their development are remarkably similar both at the morphogenetic and molecular levels (Mikkola and Millar, 2006; Mikkola, 2007). The development of skin appendages are regulated by reciprocal and sequential interactions between the ectodermal epithelium and the mesenchyme that can originate either from the mesoderm (hair and mammary gland) or the neural crest (tooth, vibrissae, and cranial hair). The epithelial–mesenchymal interactions are mediated by a small number of signalling pathways including: fibroblast growth factors, sonic hedgehog, tumour necrosis factor and transforming growth factor-β families and Wnt signalling, and their downstream transcription factors that are required for the development of these skin appendages (Mikkola, 2007). The molecular signals of growth and transcription factors are widely conserved across many species as well as across distinct appendages (Chuong, 1998; Mikkola and Millar, 2006; Mikkola, 2007).

1.2 The Hair Follicle – Epidermal Derivative of the Skin

1.2.1 Importance and Functions

The hair follicle (HF) is very complex but extremely organised structure. The HF is an attractive investigative model for studying major biological phenomena, in part due to the HF representing many human physiological attributes (Schlake, 2007; Stenn and Paus, 2001; Tobin, 2005) and has the exceptional ability to undergo growth, involution and quiescence, through an entire life time. The HF has many important aspects of mammalian biology including organogenesis, cell-cell interactions, tissue growth, regeneration and aging (Kaufman, 1996; Stenn and Paus, 2001, Botchkarev and Paus, 2003).

The HF is one of the defining features of mammalian species. Even though, hair is commonly dismissed as being of superficial importance. The HF is in fact one of human biology's most fascinating structures (Philpott and Paus, 1998; Paus and Cotsarelis, 1999; Tobin, 2005). The human hair plays an essential role in life, where the psychological importance of the hair in humans is in inverse ratio to its physical function (Ebling, 1976). The principle function of the HF is to produce a hair shaft or a hair fiber. Even though, this is also true for other mammals, in the case of humans, the hair shaft acts as a physical medium of social communications. Indeed, scalp, facial and body hairs are the only body parts an individual can shape for a person's appearance and self-image, in a social and sexual contexts. In most societies hair is seen as a sign of youthfulness (Forsland, 2000; Stenn and Paus, 2001).

The human species is frequently considered as the 'naked ape', and this ‘nakedness’ has interested researchers for many years as to why this was the case. In February’s Edition (2010) of the Scientific American magazine, an interesting article was written on why humans had evolutionary favoured to loss their hair compared to our closely living relatives, the chimpanzee. It was suggested that initial loss of hair was favoured when ‘global cooling’ in central and eastern Africa starting occurring, where ancestral humans lived (Rogers *et al.,* 2004). Human ‘nakedness’ may have lead to evolutionary acquisition of pigmentation. The identification of human melanocortin 1 receptor (MC1R) gene was shown to be crucial for melanocyte stimulating hormone (MSH) to produce skin pigmentation (Suzuki *et al.,* 1996). MC1R is believed to have originated around 1.2 million years ago in Africans, around the time where humans starting losing their hair around their body and acquiring ‘modern body proportions’ for walking, running and hunting (Rogers *et al.,* 2004).

The functions and types of the HF differ from site to site on the body. They produce hair shafts differing in size, shape, and colour. Coarse body hair are referred to as terminal hair and are found in 'hairy' areas such as the scalp, eyebrows, legs, arms, body and eyelashes in both sexes of adults. Fine and short, non-pigmented hair are refered as vellus hair, and are found on parts of the body, which are generally considered 'hairless', such as a child's cheek. These types of hair are also found in male pattern baldness (androgenic alopecia) (Randall, 1994; Stenn and Paus, 2001; Tobin *et al.,* 2003).

These variations in hair types and the distribution of these hairs on mammals have many important functions. These functions include: camouflage, thermoregulation, sensory receptors and protection from the elements and dispersion of sweat gland products (pheromones) (Oro and Scott, 1998; Paus and Cotsarelis, 1999). Thermoregulation is one of the important features of hair (heat retention) for animals, and in humans the scalp hair represents this attribute. Hair provides camouflage for hunting animals, and provides protection from abrasions, trauma, UV and acts as an insulator against cold and heat. Loss of or abnormal hair growth would be disastrous for animals, as these thermoregulatory/protective attributes of hair would be lost and survival chances would be dramatically reduced (Johnson, 1972; Abell, 1994; Stenn and Paus, 2001; Higgins *et al.,* 2009; Vogt *et al.,* 2009).

In humans, the hair is seen as of less biological importance, as loss of hair by biological or congenital abnormalities does not lead to any reduction in health of the individual or reduction in life expectancy. Even though, hair is not essential for the survival of humans, hair loss and/or excessive hair growth in men and women has been identified to be at the root of some serious psychological distress and can affect quality of life (Abell, 1994). Therefore, not surprisingly, the demand for drugs that alter hair growth has led to a multi-billion pound industry. However, relatively few drugs have been able to effectively treat HF disorders (Paus and Cotsarelis, 1999).

1.3 Hair Follicle Anatomy

The HF is a unique and is one of most complex mini-organs in mammal (**Fig. 1**). The mature HF is multi-layered structures, which consists of mesenchymal and epithelial compartments (Whiting, 2004). The hair bulb consists of the hair matrix keratinocytes, which are rapidly proliferating cells, surrounding the dermal papilla (DP), the "command centre" of the HF. The DPs communicate directly with dermal sheath cells, also referred to as connective tissue sheath, which are another type of follicular fibroblast cell populations found in the HF (Alonso and Fuchs, 2006).

The fully developed HF can be divided into three regions including the infundibulum, isthmus and the inferior segment. The infundibulum and isthmus regions of the HF are known as 'permanent' portion, while the inferior segment is a 'cycling' portion, which is continuously remodelling in each hair cycle. The infundibulum region of the HF is a continuation of the epidermis, extending from the surface of the skin to the point of the sebaceous gland duct opening to the hair canal. The isthmus portion extends from the duct of the sebaceous gland to the exertion of the arrector pili muscle (APM). The inferior segment contains the suprabulbar area and the bulb (Randall and Botchkareva, 2009; Vogt *et al.,* 2009).

The suprabulbar area of the follicle is located below the isthmus and above the bulb, and comprised of three layers from outermost to innermost: outer root sheath, inner root sheath, and hair shaft. The hair bulb contains highly

proliferative keratinocytes, and pigment-producing melanocytes (Slominski and Paus, 1993; Dawber and Messenger, 1994). These keratinocytes move up and differentiate into the hair shaft and inner root sheath (IRS). The DP contains specialised fibroblast-type cells embedded in an extracellular matrix and are separated from the hair matrix by a basement membrane (Jahoda and Oliver, 1990). The HF is separated from the dermis by the dermal sheath, which is made up of a fibroblast and a basement membrane (Nutbrown and Randall, 1995; Schneider *et al.,* 2009).

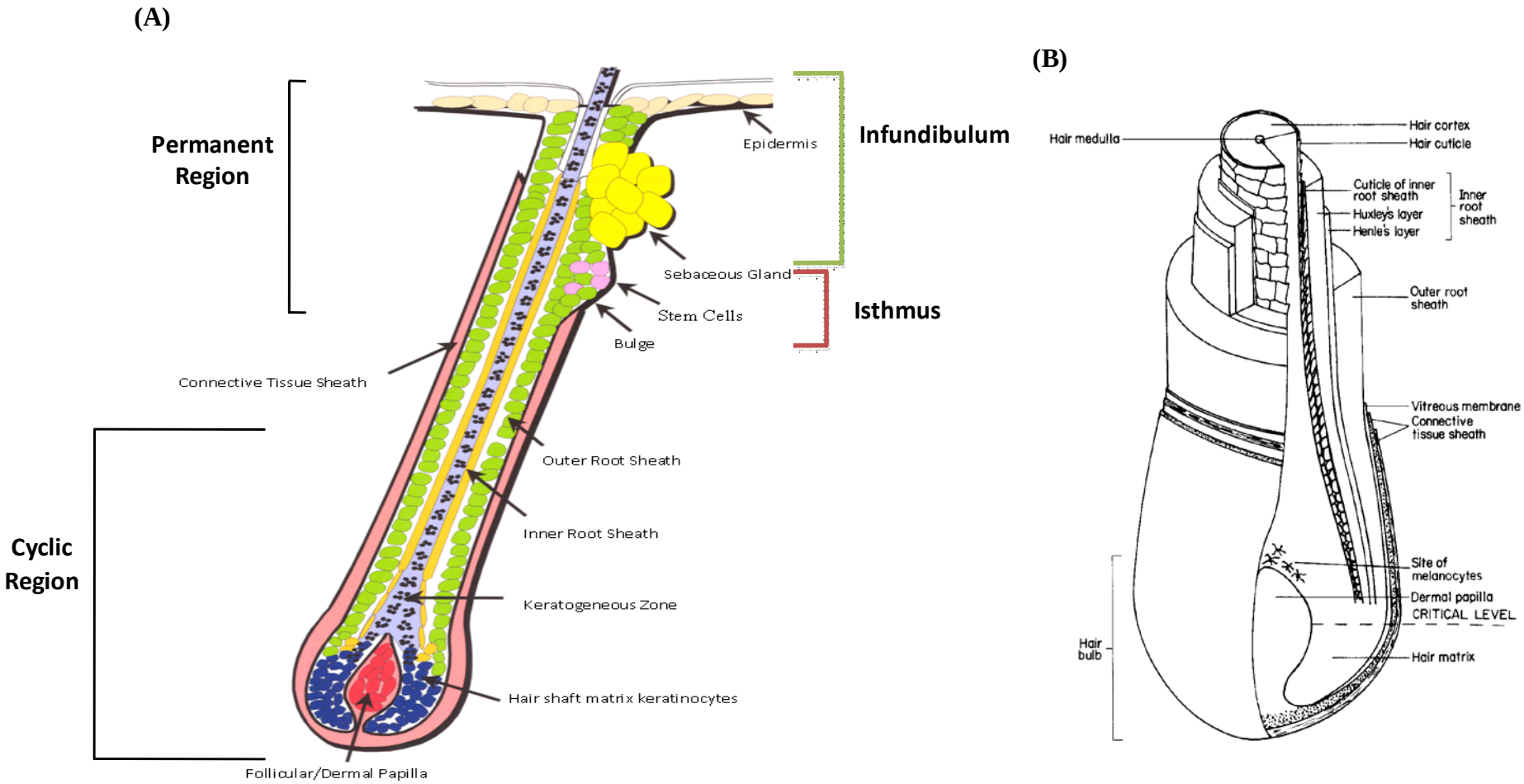


Figure 1: Hair Follicle Structure. A) Illustrative representation of a HF at anagen VI showing the different compartments. B) Illustrative representation of a hair bulb region. The hair follicle, sebaceous gland and arrector pili muscle, form the so-called pilosebaceous unit. The fibrous sheath and the epithelial compartments of the outer and inner root sheaths form concentric layers, which surrounds the hair shaft. Hair growth results from the proliferative activity of matrix keratinocytes in the bulb, which is in close proximity to the dermal papilla. The dermal papilla is a condensed "ball" of specialized mesenchymal cells with important inductive properties. (Figure 1A adapted from Margel *et al.,* 2001, Figure 1B reproduced from Randall, 1994).

1.4 Hair Shaft

The hair shaft consists of three layers: an outer cuticle, central cortex and a medulla (only in terminal hairs) (Harkey, 1993). The medulla expresses a specific hair keratin, hHa7, which has been shown to be regulated by androgen levels (Jave-Suarez *et al.,* 2004).

The main structure, forming the bulk and strength of the hair shaft, is called the cortex, which is made up of long keratinized cells, which are formed into long fibers (Harkey, 1993). The progenitor cells of the hair cortex are a group of highly mitotically-active matrix keratinocytes cells found adjacent to the distal portion of the follicular papilla (Dawber and Messenger, 1994). The hair shaft cuticle is formed by layers, which interlock with opposing layers of the IRS, which allows the hair shaft and the IRS to move upwards together (Rogers, 2004). During hair shaft growth, cortical cells emerging from the proliferative keratinocytes rapidly undergo a differentiation program and begin to synthesise large amounts of hair-specific keratin proteins (Langbein *et al.,* 2005). Differentiation of keratinocytes leads to the formation of the hair, which involves the co-ordinated activation of many hair specific structural genes (Powell and Rogers, 1997). The major proteins synthesised in the hair shaft are the keratin intermediate filament (IF) and keratin-associated proteins (KAP). Keratin IF (KIFs) are composed of type I and type II proteins, which are co-expressed in specific pairs (Steinert and Roop, 1988). The hair shaft expresses an array of hair specific keratins and their genes are activated early in hair shaft formation (Kaytes *et al.,* 1991; Powell *et al.,* 1992; Tobiasch *et al.,* 1992; Winter *et al.,* 1994; Rogers *et al.,* 1997; Langbein *et al.,* 2002). The hair shaft lineages have been shown to be extremely sensitive to Wnt signalling, as mature HFs express high levels of Lef-1 in the precursor hair

matrix cells (DasGupta and Fuchs 1999). Excessive expression of stabilized β-catenin has shown to lead to uncontrolled cell proliferation of hair matrix cells, which results in hair matrix derived tumour formation (pilomatricomas) in mice and human skin (Gat *et al.,* 1998; Chan *et al.,* 1999; Millar 2002).

1.5 The Inner Root Sheath

IRS is made of three layers: the cuticle, Huxley's and Henle's layers. The IRS is adjacent and bound to the growing hair shaft and is responsible for the surface topography of the hair fibre (Montagna and Parakkal, 1974; Swift, 1977; Ito, 1990; Rogers, 2004). These cells originate from hair matrix keratinocytes in the hair bulb and move upward parallel to the hair axis (Ito, 1986). All layers of the IRS undergo differentiation (keratinisation) but at different rates: firstly, the Henle layer, then the Huxley layer and finally, the cuticle (until half way up the follicle) (Montagna and Van Scott, 1958).

Complete keratinisation and differentiation of the IRS happens above and before the layers of the growing hair within it, therefore controlling the direction of hair growth. Keratinisation of IRS continues by increasing the production of trichohyalin (Dawber and Messenger, 1994). Trichohyalin is a structural protein that is produced and retained in the cells of the IRS (approximately one third of all protein in the IRS). Trichohyalin can be used to determine the presence/absence of IRS during HF development (Fietz *et al.,* 1993; Steinert et al., 2003).

As the cells of the IRS cuticle keratinise, the cells become thinner and overlap in a manner opposite to the direction of follicle growth. This overlapping permits the

IRS cuticle to form a template for the opposing cuticle of the hair cortex and serve to anchor and direct the growth of the hair. At the level of the HF corresponding to the sebaceous gland duct, the IRS cells degenerate, via process of tricilemmal keratinisation, which involves the lysosomal cysteine protease, catepsin-L (Tobin *et al.,* 2002), thereby releasing the hair shaft, which continues to move up and eventually protrude through the epidermis (Bertolino and O'Guin, 1994; Alonso and Fuchs, 2006). The IRS also functions as a physical barrier, which separates the hair shaft from the outer root sheath (ORS), which creates the external concentric layer of epithelial cells in the HF (Schlake, 2007). Research studies have shown that together with Henle cells and Huxley cells, the IRS ensures the maintenance of a constant desmosomal anchorage of the companion layer required for an optimal moulding and guidance of the growing hair (Rogers, 2004).

The IRS lineages have been shown to express a variety of structural keratins, which are required for its mechanical strength. However, little was known about the regulation and function of the IRS and its importance in HF development, until recently. Among the large number of transcription and growth factors known to be expressed in the skin, only a few have been functionally linked to the IRS and HF dsevelopment. These include the CCAAT displacement protein (CDP), GATA-3 transcription factor (Ellis *et al.,* 2001; Kaufman *et al.,* 2003) and BMPs (Kulessa *et al.,* 2000).

The *Cutl1* gene encoding CDP is a transcription repressor in diverse processes. CDP is expressed in the progenitors and cell lineages of the IRS. *Cutl1* deficiency leads to the reduction in size of the growing IRS, accompanied by

deregulated transcription of Sonic hedgehog (Shh), followed by the cyst formation (Ellis *et al.,* 2001).

GATA-3 is a transcription factor, which has a crucial regulatory role in IRS differentiation and is expressed in the Huxley's and cuticle layers of the IRS. Ablation of GATA-3 expression has been shown to lead to the structural changes in the IRS. In addition, GATA-3 deficient follicles are no longer spatially aligned and the hair shaft develops as an overdeveloped mass of irregular medulla, cortical, and cuticle cells. This demonstrates the importance of GATA-3 in these two layers of IRS, which are critical for the proper differentiation and/or organization of the hair shaft (Kaufman *et al.,* 2003).

BMP signalling has also been shown to be important for proper IRS differentiation. By overexpressing BMP antagonist noggin (under msx-2 promoter) in the HF, it was shown that differentiation of the IRS was severely impaired (Kulessa *et al.,* 2000).

Specialized Huxley cells of the IRS have previously been termed "Flügelzellen", in translation meaning "winged cells", due to their characteristic foot processes. It has been suggested that together with the fully differentiated Henle cells, Flügelzellen cells ensures the maintenance of a constant desmosomal anchorage of the companion layer along the whole IRS and allows the diffusion of nutrients between cells (outer to inner cells) (Clemmensen *et al.,* 1991; Langbein *et al.,* 2002).

The companion layer has close association with the Henle layer rather than ORS and therefore was termed as “companion cell layer” (Pinkus, 1958; Langbein *et al.,* 2002). It was been shown that the cells of the adjacent companion layer, which forms a functional unit with the IRS expresses at least four different keratins (Krt) including Krt6, Krt75 (Krt6hf), Krt16 and Krt17 (Winter *et al.,* 1998; Langbein *et al.,* 2002; Langbein *et al.,* 2004). The high number of expressed keratins in the companion-IRS unit could be due to keratins providing a dense and stabilizing structure. This is required for the function of the IRS-companion unit in the moulding and guidance of the growing hair (Langbein *et al.,* 2004).

1.6 The Outer Root Sheath

The ORS is a non-keratinising proliferative cellular layer of the HF, which is continuous with the epidermis (Tanaka *et al.,* 1998; Forslind, 2000). The ORS emerges proximally in the middle of the anagen bulb and continues distally to combine with the infundibulum. The ORS is established during the early stages of anagen by the downward migration of the regenerating epithelium and then sustains itself independent of the bulbar matrix by basal cell growth (Reynolds and Jahoda, 1991, Vogt *et al.,* 2009). Below the sebaceous gland, slow cycling, relatively undifferentiated stem cells reside within a niche known as the bulge (Cotsarelis *et al.,* 1990).

Cells in the ORS express an array of keratins, for example, Krt5, Krt6, Krt14 and Krt17 (Patelevey *et al.,* 1997; Langbein *et al.,* 2004). Cells in the ORS also express adhesion molecules, growth factors, and growth factor receptors that are

distinctive from those expressed by epidermal cells (Chuong *et al.,* 1998; Lyle *et al.,* 1998; Danilenko *et al.,* 1996).

In addition to the epithelial cells, other types of the cell are present in the ORS. The ORS of the HF contains amelanotic melanocytes, located between the regions of the hair bulb and the bulge of the ORS (Botchkareva *et al.,* 2001, Takada *et al.,* 1992). In human HF, Langerhans cells (dendritic antigen-presenting cells) have been found in the infundibular epithelium, including the follicular bulge, and rarely in the epithelium below the entry of sebaceous glands (Moresi and Horn, 2006). Merkel cells are specialized neurosecretory cells, located in close proximate to the bulge region of the ORS (Kim and Holbrook, 1995).

1.7 Hair Follicle Stem Cells

The functional importance of stem cells in the HF have been directed towards their roles in tissue homeostasis (Snippert *et al.,* 2010), HF regeneration (Rhee *et al.,* 2006), and wound repair (Ito *et al.,* 2005; Levy *et al.,* 2005; Levy *et al.,* 2007). Stem cells are characterized by their slowly cycling, quiescence nature and their location within a niche, known as the bulge (Cotsarelis *et al.,* 1990; Fuchs *et al.,* 2004; Lowry *et al.,* 2005). The niche is needed to preserve stem cells ability to self-renew and remain undifferentiated over a lifetime (Cotsarelis *et al.,* 1990; Oshima *et al.,* 2001; Taylor *et al.,* 2000). Recently, it was shown that slow-cycling bulge cells are formed during embryonic skin development (Nowak *et al.,* 2008). In both neonatal and adult mice, bulge restricted cells are typified by certain key stem cell markers including Sox9, Lhx2, NFATc1, Tcf3, cytokeratins 15, 19, CD 34 (in mouse), CD 200 (in humans), leucine-rich G-

protein-coupled receptor-5 (Lrg5) and Lrg6 (Lyle *et al.,* 1998; Merrill *et al.,* 2001; Li *et al.,* 2003; Blanpain *et al.,* 2004; Tumbar *et al.,* 2004; Ma *et al.,* 2004; Morris *et al.,* 2004; Vidal *et al.,* 2005; Nguyen *et al.,* 2006; Rhee *et al.,* 2006; Horsley *et al.,* 2008; Jaks *et al.,* 2008; Nowak et al., 2008; Snippert *et al.,* 2008).

Initial evidence that skin stem cells can differentiate into HF lineages, was demonstrated by targeting mouse stem cells. It was shown that the generation of all epithelial cell types within the intact follicle during normal HF cycling requires stem cell differentiation (Morris *et al.,* 2004). Subsequent studies revealed that, in both embryonic and adult skin, bulge stem cells and their transient amplifying (TA)-progeny contribute to HF regeneration but not to the maintenance of the interfollicular epidermis (Morris *et al.,* 2004; Ito *et al.,* 2005; Levy *et al.,* 2005).

At the onset of anagen growth phase, the cycling portion of the HF regenerates and undergoes a new round of hair fiber growth. This process requires a reservoir of stem cells, which reside in the lowest permanent portion of the follicle. The new downward hair growth occurs adjacent to the existing club hair, which will eventually be shed. Bulge cells less frequently divide than other epithelial cells in the skin, but these stem cells are activated at anagen to generate a new HF (Cotsarelis *et al.,* 1990; Taylor *et al.,* 2000; Blanpain *et al.,* 2004; Fuchs, 2007; Blanpain and Fuchs, 2006; Blanpain, 2010).

There are two sub-compartments of the lower follicle that exist in the resting period (telogen): the CD34 positive, slow-cycling bulge cells and the secondary hair germ (SHG) cells. SHG cells are a small cluster of P-cadherin-enriched cells

that forms at the end of catagen and separates bulge from underlying DP (Lyle *et al.,* 1999; Muller-Rover *et al.,* 1999; Panteleyev *et al.,* 2001).

The HF transition from telogen to anagen requires regeneration of HF characterized by activation of cell proliferation in the proximal follicular epithelium in the SHG and the bulge (Cotsarelis *et al.,* 1990; Cotsarelis and Miller, 2001; Oshima *et al.,* 2001). The identification of elevated Lef-1 nuclear staining in the SHG cells, initially suggested the importance of TCF3/Lef-1-mediated target genes in stem cell activation and subsequent anagen induction (DasGupta and Fuchs, 1999). Subsequently, Greco and colleagues (2009) demonstrated that at the end of telogen, the SHG stabilizes β-catenin and initiates mitogen-activated phosphokinase (MAPK) signalling. This suggests a two step mechanism of activation of stem cells during telogen-anagen transition (Greco *et al.,* 2009).

Stem cells label retaining ability was utilized by Fuchs and colleagues (2004), where they analyzed the extent of bulge stem cell involvement in giving rise to not only cells of the HF but also to epidermal cells. Using keratinocyte-specific Tet-^{off}VP16 mice, in which only bulge cells retained fluorescence, they were able to show that during anagen phase of the hair cycle, newly formed GFP-positive stem cell populations derived from the bulge stem cells, were seen to form the ORS, the hair matrix cells and IRS indicating their origins in the bulge of the ORS. In response to wounding of the skin, these GFP-labelled stem cells exited the bulge, migrated, and proliferated to repopulate the infundibulum and epidermis (Tumbar *et al.,* 2004).

Lgr5, a marker of intestinal stem cells, was also shown to be expressed in actively cycling cells in the bulge and SHG cells of telogen HFs and in the lower ORS of anagen HFs. Lgr5 positive cells represent actively proliferating and multi-potent stem cell population, which is able to give rise to new HFs and maintain all cell lineages of the HF (Jaks *et al.,* 2008).

Lgr6, a closely related gene of Lrg5 in the mammalian genome, was also identified as a possible stem cell marker (Van Loy et al., 2008). Recently, Lgr6 was shown to be one of the earliest expressed embryonic hair placodes markers along with Shh (Iseki *et al.,* 1996) and Sox9 (Vidal *et al.,* 2005). In adult HFs, Lgr6 expressing cells were shown to reside in a previously uncharacterized region directly above the HF bulge. Lgr6 positive cells expressed none of the known bulge stem cell markers, whereas Lrg5 expressing cells were strongly enriched for bulge markers such as CD34. Transplantation of Lgr6 positive stem cells from HFs of first telogen into the backs of nude mice resulted in reconstitution of fully formed HFs, interfollicular epidermis and sebaceous gland. This study identified Lgr6 as a marker for a distinct sub-population of stem cells, which gives rise to all lineages of the HF (Snippert *et al.,* 2010).

Lhx2, a transcriptional factor, which is a downstream target of Shh, was one of the first stem cell markers found to be expressed specifically in the embryonic placodes as well as in stem cells of the bulge region of post-natal HFs. Lhx2 has been shown to have a key role in HF stem cell maintenance and activation. Loss-of-function mutations of Lhx2 has shown to lead to formation of fewer number of HFs, but with the presence of more active stem cells, which are

unable to maintain stem-cell behaviour in mice (i.e., quiescence and slow cycling) (Rhee *et al.,* 2006; Fuchs, 2007).

1.8 Hair Bulb

The hair bulb consists of epithelial and mesenchymal components, the hair matrix and the DP, respectively. The HF bulb can be divided into two regions by the Auber line (Auber, 1952). The lower region of the hair bulb consists of undifferentiated, rapidly proliferating matrix cells, and the upper segment consists of differentiating, limited proliferative activity of matrix cells. The epithelial region in the bulb where keratinocytes proliferate rapidly is known as the critical region or the hair matrix zone (Orwin, 1979). The hair matrix also contains a number of dendritic melanocytes, which supply melanin pigment to the hair shaft (Dawber and Messenger, 1994; Slominski and Paus, 1993; Slominski *et al.,* 2004).

The hair matrix keratinocytes in mice express a variety of different keratins including Krt 14, Krt16, Krt17, Krt25, Krt27, and Krt28. Their expression extends from the bulb region up to the points of terminal differentiation in the IRS (Langbein *et al.,* 2004). K17 expression was seen initially in the hair bulb, localised to keratinocytes in the developing epithelial hair bulb to the unilateral disc. With advancing anagen stage the expression of keratin 17 spreads along the proximal ORS (Panteleyev *et al.,* 1997).

The production of hair involves the proliferation of epithelial cells in the bulb region, which leads to the migration and the differentiation of these cells leading

to the formation of the hair shaft and IRS (Cotsarelis, 1997; Rogers, 2004). When hair is formed by rapidly proliferating keratinocytes in the bulb, it engages with melanocytes, which deposit their melanin into the hair shaft (Cesarin 1990; Slominski *et al.,* 1994; Tobin and Bystryn, 1998; Slominski *et al.,* 2004; Randall and Botchkareva, 2009).

1.9 Dermal Papilla

The DP, also known as the follicular papilla (FP), is a population of specialized mesenchymal-derived cells forming the connective tissue compartment, located at the base of the HF (Messenger *et al.,* 1991; Hardy, 1992; Philpott and Paus, 1998; Paus and Cotsarelis, 1999; Matsuzaki and Yoshizato, 1998; Millar, 2002). The DP is believed to be one of the most important drivers to instruct the HF to grow and form a particularly sized and pigmented hair shaft (Jahoda *et al.,* 1984; Matsuzaki and Yoshizato, 1998; Van Neste and Tobin, 2004).

DP is responsible for establishing, maintaining proliferation and differentiation of hair matrix cells during hair growth cycle. Several experiments have shown that the DP has powerful inductive properties. Transplant studies have indicated that DP cells are capable of inducing follicular development and the formation of the hair shaft when placed in a non-hair containing skin (Jahoda and Morris, 1984; Jahoda and Oliver, 1984; Jahoda *et al.,* 1993). DP keeps a close-contact with the HF epithelium throughout the entire hair growth cycle (Young, 1980), which induces the HF epithelium to create a functional and cycling HF (Hardy, 1992; Oro and Scott, 1998; Matsuzaki and Yoshizato, 1998; Kishimoto *et al.,* 2000). During the hair cycle, the papilla reaches maximal size by anagen VI, but

decreases by 25% in size during early periods of sustained hair production. This cell loss was shown not to be associated with intra-follicular papilla apoptosis, but instead it was strongly suggested that fibroblasts migrate out of the late anagen/early catagen papilla and re-enter the proximal connect tissue sheath (CTS) (Tobin *et al.,* 2003).

The DP is a crucial source of paracrine factors vital for hair growth. The DP provides essential stimuli, for example, noggin, which exerts a hair growth-inducing effect by antagonising bone morphogenetic protein (BMP) signalling activation of the BMP receptor IA expressed in the follicular epithelium (Botchkarev *et al.,* 2001). Hepatocyte growth factors (HGF) expressed in the DP and its receptor *c*-met, which is expressed in the hair matrix, have been shown to be crucial for hair growth. Transgenic mice overexpressing HGF exhibit accelerated HF development (Lindner *et al.,* 2000). Stem cell factor (SCF) is critical for proliferation, differentiation, and melanin production by follicular melanocytes expressing its receptor c-kit (Botchkareva *et al.,* 2001). Insulin growth factor (IGF) produced by the DP also acts as an important morphogen in the HF (Rudman *et al.,* 1997). In addition, many other molecules are produced by the DP for follicle induction, cycling and pigmentation (Paus and Cotsarelis, 1999).

The DP also displays strong alkaline phosphatase activity during the hair growth cycle (Handjiski *et al.,* 1994). The activity of alkaline phosphatase has been used as an indicator to detect the presence of DP during the hair cycle. Alkaline phosphatase activity in DP and dermal sheath has been shown to reach its

highest level in early anagen, and its expression was seen to decrease after mid-anagen stage of the hair cycle. These temporal and spatial changes in alkaline phosphatase activity coincide with the hair-inductive property of DP and dermal sheath (McElwee *et al.,* 2003; Lida *et al.,* 2007). Even though, the role for alkaline phosphatase in hair growth cycle remains unclear, it has been shown that hair growth is reduced when alkaline phosphatase is inhibited (Lida *et al.,* 2007).

1.10 The Connective Tissue Sheath

The connective tissue sheath (CTS), is also known as the dermal sheath, joins with the base of the DP via a narrow line of tissue known as the basal stalk. This broadens distally to produce a rounded end to the follicle, called the basal plate. The basal plate encloses the remaining follicle along its whole length, which includes the sebaceous gland and integrates into the papillary layer of the skin dermis (Oliver, 1966; Jahoda and Oliver, 1990). CTS runs along the epithelial ORS separated by a thick and specialized basement membrane structure known as the glassy membrane. Dermal sheath cells act as a “pool” of papilla cells in injury situations, and may interchange with papilla cells as part of and during the catagen/telogen/early anagen phases of the hair growth cycle (Jahoda and Reynalds, 2001). These interchanges show significant hair cycle-associated plasticity and these cell interchanges are likely to be important during clinically important HF transformations, for example: during vellus-to-terminal and terminal-to-vellus and/or during androgenetic alopecia (Tobin *et al.,* 2003).

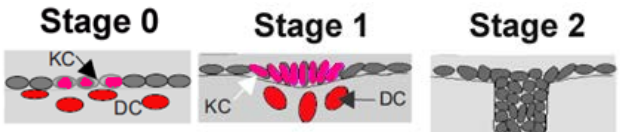
1.11 Hair follicle morphogenesis

HF morphogenesis occurs in eight distinctive morphological stages (**Fig. 2**) characterised by the downward movement of developing HFs, the positioning of the DP, the presence and position of the IRS and melanocytes. HF begins to develop from 9th to 12th week of estimated gestation age (EGA) in human skin embryo. The HF is first found in the chin, eyebrows and upper lips of the developing embryo (Breathnach and Smith, 1968) and then HFs progressively cover the entire body (Hashimoto, 1970; Philpott and Paus, 1998; McElwee and Sinclair, 2008).

The early stages of HF morphogenesis (stages 0-3), is initially characterised by a single layer of basal keratinocytes (stage 0), while over time this basal layer of the epidermis begins to thicken forming a hair placode (stage 1) (hair germ) (Breathnach and Smith, 1968; Hashimoto, 1970; Paus *et al.,* 1999). The increasing number of dermal fibroblasts gathering beneath the hair placode has been shown to be crucial for HF morphogenesis (Oro and Scott, 1998; Philpott and Paus, 1998; Panteleyev *et al.,* 2000).

The HF continues to grow downwards, with increasing number of keratinocytes in the developing HF. The developing HF is characterised by the cap like covering of dermal fibroblasts on the bottom of the ectoderm (stage 2). Subsequent stages of HF morphogenesis are characterised by the continuation of the HF to grow downwards, with the developing HF now consisting of multiple layers of keratinocytes that become concentrically oriented towards the follicular axis (stage 3) (Robins and Breathnach, 1969). This is followed by the developing

HF resembling an immature HF, with a bulb like thickening, the presence of the IRS cone and the increased surrounding of the DP by the keratinocytes of the developing hair bulb (stage 4). Stages 5- 7 of HF morphogenesis are characterised by the appearance of the APM, presence of melanin granules, elongation of the IRS, the initial development of the ORS and sebocytes, and the visibility and enlargement of the bulge (stage 5). Stages 6-7 of HF morphogenesis characterised by the formation of the hair canal, by completely enclosed the DP by the hair bulb, by presence of sebaceous gland, and by IRS containing a hair shaft. The hair shaft continues to move upwards and protrudes into the hair canal (exclusively stage 7). During the final stage of HF morphogenesis (stage 8), HF is morphologically similar to mature anagen VI HF. During this stage the HF reaches its maximal length and reaches the subcutaneous layer (Parakkal, 1969; Paus *et al.,* 1999).



Interactions between activators and inhibitors for HF induction

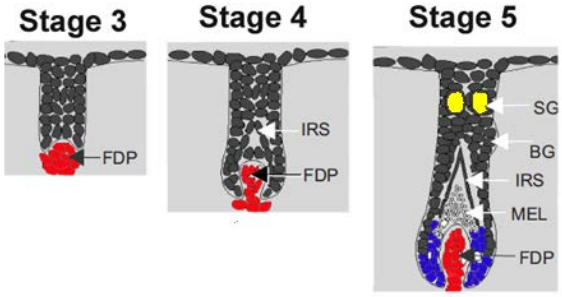
Key signalling:
Wnt/β-catenin signalling, BMPs and Shh

Promoters of Hair placode development:
β-catenin, Wnt10b, Lef-1 transcription factor, Noggin, EDAA1/EDAR/NF-κβ

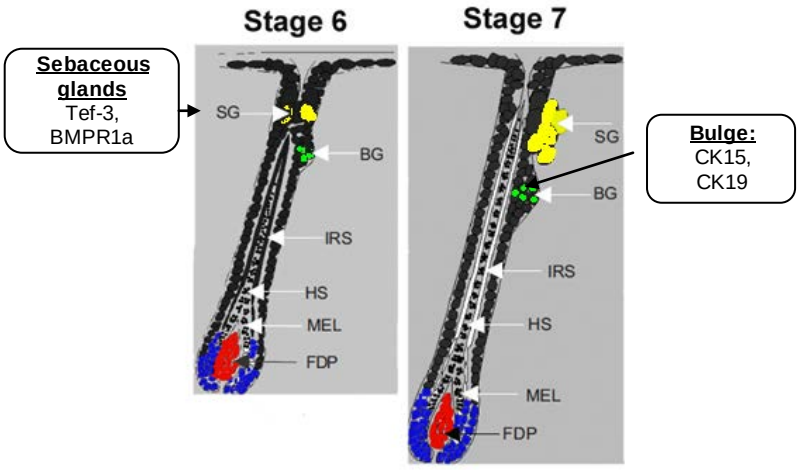
Inhibitors of hair placode development:
KGF, EGF. BMP4, p75NTR, Notch-1, Dickopf 4

Proliferation of dermal hair germ cells:
Shh/Gli2, TGF-βR-II, PDGF-A,

Formation of dermal papilla:
Shh, Wnt5a, Lef-1, Noggin, p75kD NTR



Proliferation of follicular epidermis:
Shh,Gli2
Polarity of hair follicle, hair shaft formation:
Wnt/β-catenin signalling, Noggin/Lef-1, Shh FGF,
Shape of hair follicle:
TGF-α/EGFR, FGFRII-IIIb
Differentiation of inner root sheath:
GATA-3, Cutl1, CK1,CK10, Trichoyalin, EGF, Notch
Differentiation of hair shaft:
Hair keratins, BMP-2, BMP-4, BMPR1a, Notch-1, Lef-1, Msx2, GATA-3, HoxC13
Differentiation of outer root sheath:
CK5, CK15
Dermal papilla:
BMP-2, BMP-4, Noggin, BMPR1a, SCF,



Dermal papilla:
BMP-2, BMP-4, Noggin, BMPR1a, KGF, HGF, SCF,

Figure 2. Illustrative representation of the hair follicle morphogenesis and the molecular signalling involved in induction and subsequent HF development. HF morphogenesis occurs in eight distinctive stages. Upperparts: The most-important developmental stages of murine hair follicles. Below: The best-studied molecular players of each stage. The early stages of HF morphogenesis characterised by a single layer of basal keratinocytes (stage 0). This stage is characterised by the balance of activators and inhibitors of hair follicle development. Subsequent exchanges in signals between the epithelial and underlying mesenchyme giving rise to the dermal papilla characterised by the thickening of the epidermis, gathering of dermal fibroblast (hair germ) and the HF keratinocytes display a vertical polarisation orientation (stage 1). The HF continues to grow downwards, with increasing number of dermal papillas (stage 2). The subsequent stages of HF development include several crucial signalling pathways for the proper formation of the HF including Shh, Wnt and BMP signalling. Stages 3-4 of HF morphogenesis: HFs continue to grow downwards, with the developing HF now consisting of multiple layers of keratinocytes (stage 3) and consisting of similar characteristic of an immature HF, with a bulb like thickening, the presence of the IRS cone. The dermal papilla is increasingly surrounded by the keratinocytes of the developing hair bulb (stage 4). The following stages of HF development (stages 5-8) are characterised by the site of the attachment of the arrector pilli muscle, presence of melanin granules, elongation of the IRS, the initial development of the ORS and sebocytes and the visibility and enlargement of the bulge (stage 5). The hair canal is visible, the DP is completely enclosed by the hair bulb, presence of sebaceous glands and IRS contains hair shaft and the hair shaft continues to move upwards and protrudes into the hair canal. The final stage of HF morphogenesis (stage 8) resembles morphologically a mature anagen VI HF. Abbreviations: KC, keratinocytes; DC, dermal cells; FDP, Follicular dermal papilla; IRS, inner root sheath; SG, sebaceous gland; BG, bulge; MEL, melanin; HS, hair shaft, BMP; Bone morphogenic protein, Wnt; Wingless, Lef; lymphocyte effector factor, EDAR; Ectodysplasin receptor, EDA; Ectodysplasin, KGF; Keratinocyte growth factor, EGF; Epidermal growth factor, TGF; Transforming growth factor, PDGF; Platelet-derived growth factor, SCF; Stem cell factor CK; Cytokeratins, HGF; Hepatocyte growth factor. Adapted from Schmidt-Ullrich and Paus, 2005; Botchkarev and Paus, 2003.

1.12 Important signalling pathways involved in hair follicle development

Each stage of HF morphogenesis is characterized by unique expression patterns of regulators, which either promote HF induction and subsequently HF development or are inhibitors of this process (**Fig. 2**). These regulators come in a broad category of growth factors and their receptors, growth factor antagonists, adhesion molecules, and intracellular signal transduction components (Botchkarev and Paus, 2003).

Studies using post-natal animals have indicated that the genetic program of HF-specific differentiation in epidermis may be initiated after receiving the mesenchymal signal or ‘‘first dermal message’’ (Hardy, 1992). This “first dermal message’’ leads to the beginning of the formation of the focal thickening in the basal layer of epidermis (hair germ or hair placode, stage 1), in which cells become oriented vertically, change their adhesion from regular epidermal keratinocytes (Paus *et al.,* 1999; Nanba *et al.,* 2000). These keratinocytes can be distinguished from regular epidermal keratinocytes by the expression of several growth factors (Wnt-10b, Eda, BMP-2), growth factor receptors (Edar, TGF-βRII, BMPR-IA), and transcription regulators (β-catenin, Lef-1, Msx-2) (Zhou *et al.,* 1995; Paus *et al.,* 1997; St-Jacques *et al.,* 1998; DasGupta and Fuchs, 1999; Headon and Overbeek, 1999; Botchkarev *et al.,* 1999; Foitzik *et al.,* 2000; Reddy *et al.,* 2001; Kusa *et al.,* 2001).

The Wnt/β-catenin/Lef-1 signalling pathway is one of the crucial pathways involved in initiating HF development. Wnt-10a, Wnt-10b, β-catenin and Lef-1 are all expressed in the epithelium of stage 1 HFs (Botchkarev *et al.,* 1999;

DasGupta and Fuchs, 1999; Huelsken *et al.,* 2001; Reddy *et al.,* 2001; St-Jacques *et al.,* 1998). The ectopic expression of Dickkopf-1 (Dkk), a Wnt-antagonist, in murine epidermis resulted in abrogation of induction of all HFs types in back skin (Andl *et al.,* 2002). The presence of Dkks at sites of Wnt signalling may implicate the 'reaction-diffusion' model, where cell fate is determined by the concentration/levels of activator and/or inhibitors of HF induction (Jung *et al.,* 1998).

Lef-1 knockout mice showed an absence of most of the HFs in back skin and lack of vibrissa follicles (van Genderen *et al.,* 1994). Transgenic expression of Lef-1 leads to ectopic HF development in oral epithelium (Zhou *et al.,* 1995; Gat *et al.,* 1998). Lef-1 has been shown to negatively regulate the expression of E-cadherin in hair germ keratinocytes and promotes the loss of their adhesion to neighbouring epidermal keratinocytes, therefore stimulating HF induction (Jamora *et al.,* 2003). Recently, it has also been shown Wnt/β–catenin signalling pathway has a pivotal role in the formation of *de novo* HFs after wounding in murine skin. Ito and colleagues (2007) showed that selective ablation of Wnt signalling leads to abrogation of wound-induced folliculogenesis (Ito *et al.,* 2007). The Wnt signalling pathway was also shown to upregulate the expression of Dkk-4 in pre-placode stage. Dkk4 is transiently expressed during the pre-placode stage of appendage formation to inhibit canonical Wnt signalling and/or laterally inhibit the surrounding epithelium from adopting a placode fate, similar to the role of Dkk1 in the mesenchyme (Andl *et al.,* 2002). This suggests that canonical Wnt signalling not only is essential for the induction of HF, but is also responsible for

the upregulation of Dkk4 in the pre-placodes, which is believed to regulate the pattern of HFs in the epidermis (Bazzi *et al.,* 2007)

Other important regulators of initiating HF development are the ectodysplasin (Eda) and its receptor (Edar), and TGF-β signalling. Eda and its receptor Edar belong to the TNF-α family. It has been shown that deletion of both Eda and Edar in corresponding mouse mutants (Tabby and Downless, respectively) is accompanied by failure in induction of primary or tylotrich HFs (Headon and Overbeek, 1999). TGF-/β-2 deficient mice showed a delay of HF development and reduced number of HFs. In contrast, overexpression of TGF-/β-2 has been shown to stimulate HF initiation in embryonic skin explants (Foitzik *et al.,* 1999).

In addition to the activators of HF induction, the skin also contains a number of inhibitors of HF induction including, BMPs, keratinocyte growth factor (KGF) and epidermal growth factor (EGF) signalling (Richardson *et al.,* 2009). During HF induction in mice, both BMP-2 and BMPR-IA are expressed in hair placode, while BMP-4 and noggin expression is seen in cells of mesenchymal condensation beneath the placode (Botchkarev *et al.,* 1999). Botchkarev and colleagues (1999) showed that neutralization of BMP-2 and BMP-4 inhibitory activity by noggin stimulates the initiation phase in HF development (Botchkarev *et al.,* 1999). Along with BMP signalling, EGF and KGF have been established as inhibitors of HF induction. KGF and EGF receptors have been shown to be markedly down-regulated in hair placodes and elevated levels of ligands for each receptor resulted in the inhibition of HF formation in embryonic organ culture (Richardson *et al.,* 2009).

Constitutive deletion of Shh has been shown to lead to arrest of HF morphogenesis at stage 2, which was accompanied by a reduced keratinocyte proliferation and appearance of rudimentary follicular papilla (St-Jacques *et al.,* 1998; Chiang *et al.,* 1999). Other important molecular signals involved in HF development include the platelet-derived growth factor-A (PDGF-A), which promotes HF development by stimulating the growth of follicular papilla. PDGF-A null HFs show smaller follicular papillae and abnormal CTS (Karlsson *et al*., 1999). Neutrophins can also regulate HF development via activation of their low affinity receptor p75 neurotrophin receptor (NTR). p75NTR expression is restricted to the DP of the developing HF. p75NTR knockout mice showed acceleration of HF morphogenesis. It was shown that signalling via p75 may control proliferation and differentiation balance in the fibroblasts of the growing DP. In addition, it was also shown that p75 NTR activation prevented early onset of FGFR-2 expression in the DP cells (Botchkareva *et al.,* 1999).

The later stages of HF development have been shown to be dependent on proper IRS and hair shaft differentiation. These processes of differentiation in the IRS and hair shaft are dependent on several crucial signalling pathways. Differentiation and normal development of the IRS has been shown to be dependent primarily on GATA-3, *Cutl1* and Foxn1. GATA-3, a transcription factor, which has important regulatory role in IRS differentiation, has been shown to lead to the structural changes of IRS when its expression is inhibited (Kaufman *et al.,* 2003). The *Cutl1* gene has been shown to expresses broadly in the IRS. *Cutl1*-deficient follicles exhibit an initial development and formation of IRS, but then both the IRS and hair shaft degenerate (Ellis *et al.,* 2001). Foxn1 knockout

or nude mice show formation of abnormal globular aggregates in the IRS and truncated hair shafts (Prowse *et al.,* 1999).

The hair shaft undergoes differentiation via several important signalling pathways. This includes the Wnt/β-catenin/Lef-1 signalling, which has a critically important role in hair shaft formation. Lef-1 knockout mice show arrest of HF development prior the beginning of hair shaft formation (Van Genderen *et al.,* 1994; DasGupta and Fuchs, 1999). Hoxc13 is a transcription regulator that has also been shown to be involved in the control of hair shaft formation. Hoxc13 overexpression in differentiating hair shaft cells resulted in fragile hairs and alopecia (Jave-Suarez *et al.,* 2002). Foxn1 transcription factor, which is mutated in nude mice, is also expressed in differentiating hair shaft keratinocytes (Meier *et al.,* 1999). Interestingly BMP signalling has been shown to be involved in controlling the expression and function of Lef-1, Hoxc13, and Foxn1 transcription factors in differentiating hair shaft precursor cells. In transgenic mice overexpressing noggin, showed downregulation of Foxn1 and Hoxc13 expression in differentiating hair shaft cells, while ectopic expression of Lef-1 was observed in the upper portion of the hair shaft (Kulessa *et al.,* 2000). Msx-2 transgenic mice have been shown to have markedly reduced number of hair matrix keratinocytes (Jiang *et al.,* 1999). In contrast, Msx-2 knockout mice show structurally abnormal hair shafts (Ma *et al.,* 2003).

A Notch signalling pathway has also been shown to have an important role in the control of hair shaft-specific differentiation in the HF. *Notch1* and its ligands Jagged1/2 are expressed in differentiating cells of the IRS and hair shaft (Kopan

and Weintraub, 1993; Powell *et al.,* 1998; Favier *et al.,* 2000; Lin *et al.,* 2000), and have been shown to maintain IRS precursor cells, which are required for the formation of IRS (Nicolas *et al.,* 2003; Pan *et al.,* 2004). The overexpression of *Notch1* in these cells leads to abnormal differentiation of the hair medulla and wavy, sheen pelage hairs (Lin *et al.,* 2000).

1.13 Hair Growth Cycle

The hair growth cycle is a uniquely reproducible and cyclic regenerative phenomenon, which occurs in a uniform manner in thousands of follicles simultaneously over the entire skin of humans and other mammals (Cotsarelis, 1997). Immediately on completion of morphogenesis, the HF resembles fully developed anagen HF and then the HF enters into a lifelong cycle of organ transformation from active growth (anagen), brief period of organ involution (catagen), followed by a relative "resting" phase (telogen) (Paus *et al.,* 1999; Paus and Foitzik, 2004).

This cyclic nature of the HF is tightly controlled by various factors and interactions between epithelial, neuro-ectodermal and mesenchymal cells (Slominski and Paus, 1993; Paus *et al.,* 1999). In humans, each active/growth or anagen phase of the hair cycle last 3-10 years mean duration. Duration of catagen is 3 week. The resting phase (telogen) is 3 month long (Paus *et al.,* 1999; Vogt *et al.,* 2009) (**Fig. 3**).

Investigations into molecular regulations of HF cycling have been widely elucidated using genetically engineered knockout mice or overexpression of a

specific gene product closely linked to an epidermal and hair phenotype (Chen and Roop, 2008; Nakamura *et al.,* 2001). It is now widely accepted that HF cycling is a result of exchanges of signalling between the epithelial and meschencymal compartments of the growing HFs (Stenn and Paus, 2001).

HF cycling is controlled by an array of signalling networks within and between the follicular epithelium and mesenchyme (Botchkarev and Fessing, 2005). These two compartments employ molecules that belong to the Wnt signalling family, TGF-β, BMPs, Shh, Fgfs, *Notch*, EGF, TNF-α, and neurotrophin families, all of which are crucial for regeneration, maintenance and cycling capabilities of the HF (Botchkareva *et al.,* 1999; Cotsarelis and Millar, 2001; Fuchs *et al,* 2001; Millar, 2002; Botchkarev and Kishimoto, 2003; Schmidt-Ullrich and Paus, 2005).

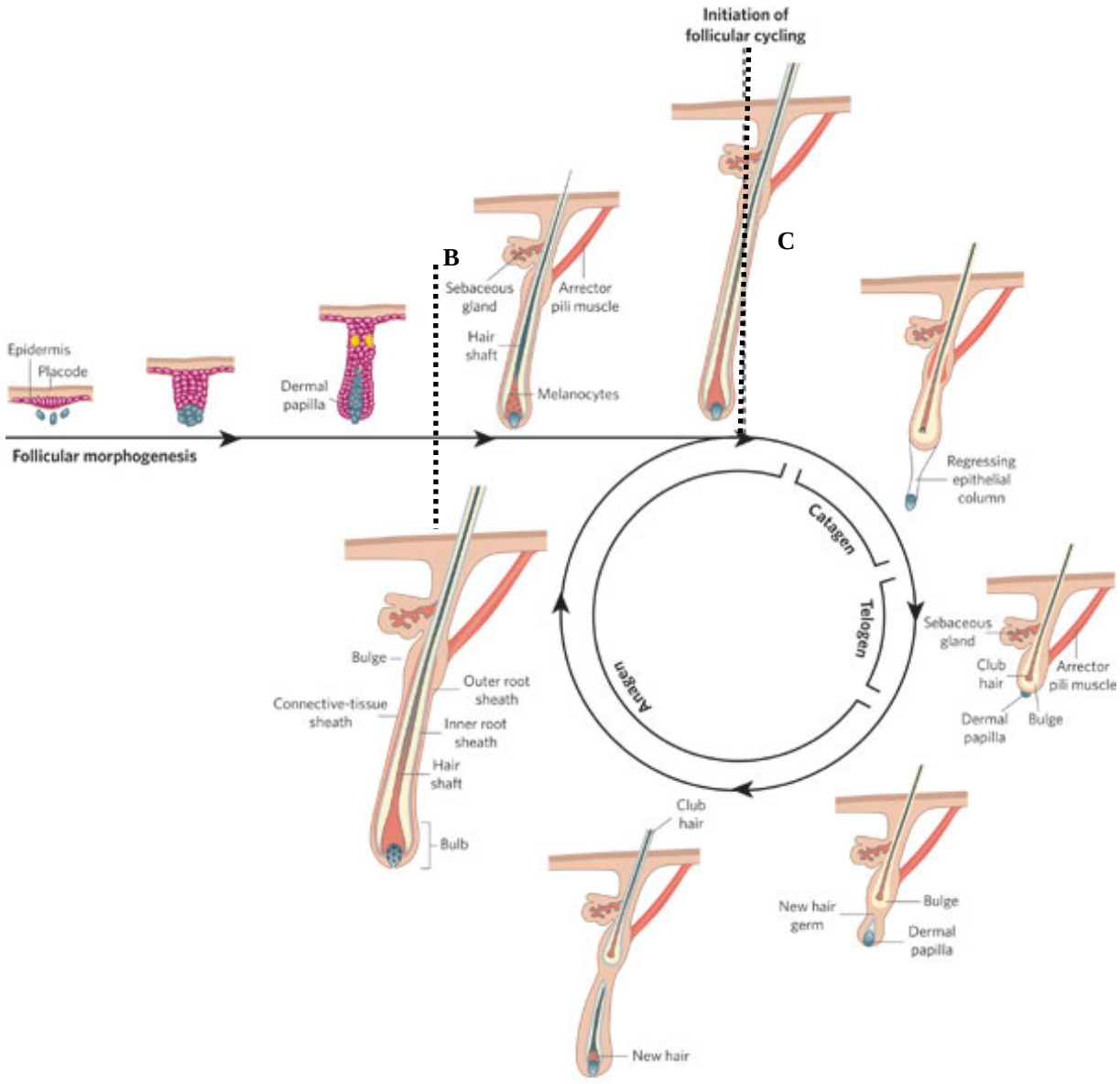


Figure 3. The hair growth cycle. A) Hair follicle morphogenesis with initial interactions/signal exchanges between the epithelial and mesenchymal cell populations. **B**) First post-natal anagen, with the hair shaft protruding through the epidermis and **C**) hair follicles then enter a regressing phase (catagen), which is characterized by tightly controlled levels of apoptosis. The dermal papilla, stem cells and secondary hair germ cells are located in close proximity to the dermal papilla, with minimal signal exchanges/activity occurring during resting phase (telogen). Activation of stem cells leads to hair growth (anagen). Adapted from Paus and Cotsarelis, 1999, and Fuchs, 2007.

1.13.1 Telogen

Telogen HFs are very short in length and characterised by a lack of pigment-producing melanocytes and the absence of the IRS. In telogen phase, the HF has reduced to about one third of its previous size and does not extend beyond the upper dermis. The epithelial cells of the lower telogen follicle do not show significant DNA or RNA synthesis, nor is there any synthesis of proteins characteristic of the anagen follicle (Vogt *et al.,* 2009).

Telogen is currently seen as more than just a period of "resting", as the epithelial 'remnants' of the telogen HF such as the bulge, SHG and compact DP are still engaging with each other. Telogen stage of hair growth cycle is being intensely investigated due to the balance between growth activators and inhibitors observed during this stage. Alterations to these growth modulators may lead to the initiation or prevention of active growth phase (anagen). Therefore local balance of growth stimulators and inhibitors in the proximal part of HFs appears to be critical for new hair growth wave initiation (Botchkarev and Paus, 2003).

The HF transition and transformation from telogen to anagen stages of the hair cycle are a unique process of programmed organ regeneration characterized by activation of cell proliferation in the follicular germinative compartment located in most proximal part of the telogen HFs close to DP (Stenn and Paus, 2001).

Telogen skin contains inhibitors of hair growth such as BMP 2/4. Botchkarev and colleagues (2001) demonstrated that the initiation of anagen requires inhibition of BMP-4/BMPR-IA signalling by noggin. In the telogen HFs, BMP4 is produced by

both the DP fibroblasts and the hair matrix keratinocytes, and interacts with BMPR-IA, which is selectively expressed in the SHG and therefore inhibiting the early onset of anagen development (Botchkarev *et al.,* 1999; Botchkarev *et al.,* 1999; Botchkarev *et al.,* 2001). In telogen HFs, keratinocytes of the SHG express structural proteins (cytokeratins 14), growth modulatory molecules (BMP-4, Shh, fibroblast growth factor 5 (FGF5)), growth factor receptors (bone morphogenetic protein receptor IA (BMPR-IA), adhesion molecules (E- and P-cadherins), and transcriptional regulators (β-catenin/Lef-1, STAT3) (Botchkarev and Paus, 2003; Botchkarev and Khisimoto, 2003).

At the end of telogen stage a new anagen phase begins as the SHG cells proliferate and move down into the dermis with the DP cells. A complete new hair bulb develops, which grows into the base of the existing resting follicle. The new hair then pushes up beside the existing one which is shed (exogen). SHG cells in telogen HFs are a small cluster of cells located between the DP and the bulge. SHG cells are a distinct population of stem cells, with slow-cycling characteristics but quicker responsiveness to activating signals compared to stems cells in the bulge. This suggests that while stem cells of the bulge maintain the process of hair growth cycle, the role of SHG stem cells is to initiate hair regeneration (Greco *et al.,* 2009).

Recently, Greco and colleagues (2009) identified Fgf7 and Fgf10 as potent activators of hair regeneration by predominately having an effect on SHG cells and not the bulge during telogen-anagen transition. They also showed levels of

Wnts transcripts and the level of β-catenin stabilisation elevated during late telogen/early anagen in the SHG cells (Greco *et al.,* 2009).

Cell proliferation in the germinative compartment of the telogen HF can also be activated by applying mechanical or chemical stimuli. An example of mechanical stimulation, which is currently used to induce and investigate the murine hair growth cycle, is plucking/removal of the hair shafts using warm wax on the back skin hair of a mouse. This would not only lead to the onset of a new hair growth cycle but also synchronising majority of HFs in this region. This allows the analysis of expression patterns of genes of interest at distinct hair cycle stages. However, there is the possibility of abnormal effects due to the wounding caused by plucking (Paus *et al.,* 1999; Paus, 1998).

1.13.2 Anagen

The anagen phase of the hair cycle can be divided into six sub-stages. The earliest changes observed during anagen initiation have been shown to occur in the cells of the DP and the SHG cells (Stenn and Paus, 2001). During mid-anagen hair progenitor cells proliferate, surround the growing DP, move downwards into the subcutaneous tissue and begin to differentiate into the hair shaft and IRS. Melanocytes located in the hair matrix show pigment-producing activity (Slominksi *et al.,* 2005), and the newly formed hair shaft begins to develop. Late anagen is characterised by the fully formed hair bulb, which engulfs the DP located deep in the subcutaneous tissue, and by emerging a pigmented hair shaft through the epidermis (Paus *et al.,* 1999; Stenn and Paus, 2001; Randall and Botchkareva, 2009; Vogt *et al.,* 2009).

There are at least three crucial signalling pathways in mice, which are known to participate in driving HFs from telogen to anagen. These three pathways include Shh, the Wnt and BMP signalling pathways. In telogen stage of the hair cycle, stabilization of β-catenin, a recognized effector of active Wnt signalling and a transcriptional cofactor for Lef1/TCF proteins (Gat *et al.,* 1998; Celso *et al.,* 2004; Lowry *et al.,* 2005; Van Mater *et al.,* 2003) is required for onset of new hair growth. During telogen, active BMP signalling is required for maintaining bulge stem cells in a relatively quiescent state (Blanpain *et al.,* 2004; Horsley *et al.,* 2008), and therefore preventing early anagen induction (Rendl *et al.,* 2008).

Induction of Shh target genes occurs in the anagen HF in response to expression of Shh. The Shh pathway is vital for maintaining the stem cell population, and regulating the development of HFs (Oro and Higgins, 2003; Athar *et al.,* 1999). Overexpression of Shh in mice was shown to accelerate HFs transition from telogen to anagen (Sato *et al.,* 1999). In addition, significance of Shh in telogen-anagen transition was shown by a single topical application of synthetic agonists of the Shh pathway, which stimulated anagen induction (Paladini, *et al.,* 2005).

Onset of Wnt signalling activation promotes several transcriptional changes in the HF, particularly in the proliferating and differentiating keratinocytes of the hair bulb (Lowry *et al.*, 2005). This activation of Wnt signalling coincides with the decrease in BMP signalling, therefore, subdividing the telogen phase into a high BMP refractory phase and a low BMP competent phase for hair regeneration and anagen induction (Plikus *et al.,* 2008). Initial evidence of the role of Wnt/β-catenin signalling in anagen induction came from a study using TOPgal transgenic mice

carrying a TCF/LEF optimal promoter upstream of a β-galactosidase gene. They showed an increase in β-galactosidase in the bulge region of the HFs at the onset of anagen (DasGupta and Fuchs, 1999). Also, the first anagen-like stage (postnatal day 12) was shown to not occur in mice in which β-catenin expression is progressively lost in the skin (Huelsken *et al.,* 2001). Wnts 10a and 10b have been shown to be expressed in postnatal HFs at anagen onset, but not in resting follicles of telogen skin (Reddy *et al.,* 2001). These data are consistent with the view that a Wnt signal activates β-catenin signalling in the bulge, thereby driving the resting follicle into active growth. These studies demonstrate a role for Wnt/β-catenin signalling during initial development of HFs as well as required for anagen induction (Van Mater *et al.,* 2003).

Activation of Wnt and Shh signalling in early anagen HFs may be due to the down-regulation of BMP signalling, which antagonizes the Wnt and Shh pathways in HFs (Baker *et al,* 1999; Botchkarev *et al,* 2001). Interestingly, BMPR-IA expression is significantly reduced in SHG keratinocytes, which show the highest rates of proliferation in early anagen HFs, while expression of the BMP antagonist noggin is increased in the follicular epithelium and in the mesenchyme (Botchkarev *et al,* 2001).

During anagen development, the hair matrix keratinocytes also express an array of growth modulatory molecules, growth factor receptors and transcriptional regulators that are required for normal hair growth, including, Shh, β-catenin /Lef-1, *c*-kit, *c*-met, Fibroblast growth factor receptor 2 (FgfR2), KGF, IGF-I receptor. The corresponding ligands of the receptors found in the hair matrix, are

expressed in the DP, such as Wnt5a, SCF, HGF, Fgf7 and IGF-1. These signalling pathways are essential for HF cycling (Philpott *et al.,* 1994; Paus and Cotsarelis, 1999; Botchkarev *et al.,* 2003; Botchkarev *et al.,* 1999; Danilenko *et al.,* 1996; Botchkarev and Kishimoto, 2003; Randall and Botchkareva, 2009).

Studies using mice that lack IGF-1 or their receptor revealed poorly developed HFs. IGF-1 has been shown to maintain and increase follicle growth *in vitro* (Philpott *et al.,* 1994; Stenn *et al.,* 1996). Mice that lack Fgf-7 have reasonably normal HFs, (Guo *et al.,* 2006) but disruption of the receptor for Fgf-7, which is also the receptor for Fgf-2, causes markedly reduced and abnormal HF formation (Werner *et al.,* 1994).

The end of the anagen phase is believed to be controlled in part by Fgf5. Fgf5 is the most powerful molecule known so far to control anagen-catagen transition (Herbert *et al.,* 1994; Pethö-Schramm *et al.,* 1996). Fgf5 is highly expressed in the HFs just before the end of anagen stage (Rosenquist and Martin, 1996). Studies have shown that mice that lack Fgf5 have an extended anagen stage, leading in the 'angora' phenotype, with hair that is 50 percent longer than normal (Sunberg *et al.,* 1997). Even in mice that lack this growth factor, the follicle still eventually enters the catagen stage, indicating that other signalling pathways are also important for the induction of catagen stage. The transition from anagen to catagen also involves EGF receptors, as mice without EGF receptors or with non-functional receptors had extended anagen stage (Hansen *et al.,* 1997).

Members of the neurophin family, including brain-derived neurotrophic factor (BDNF), NT-3 and NT-4 have been also shown to be upregulated in the proximal HF epithelium just before the onset of catagen, whereas their high affinity receptors TrkB and TrkC are expressed in the DP during late anagen-early catagen (Botchkarev *et al.,* 1998; Botchkarev *et al.,* 1999). Transgenic mice, overexpressing either NT-3 or BDNF, under K14 promoter, showed premature catagen development. BDNF and NT-3 leads to downregulation of SCF and Vascular endothelial growth factor (VEGF) secretion by DP cells *in vitro*. This could partially explain neutrophin-induced anagen-catagen transition molecular mechanisms (Tobin *et al.,* 1999; Botchkarev and Paus, 2003).

1.13.3 Catagen

The transition of actively growing HF into the stage of spontaneous involution (catagen) is a biological process, which varies in different body regions and determines the diversity of fur seen in animals and humans. This regression of the HF in catagen is characterized by 1) a cessation of proliferation and differentiation of hair matrix keratinocytes; 2) termination of melanogenesis, and 3) activation of massive apoptosis in the hair matrix (Slominski *et al.,* 1994; Lindner *et al.,* 1997; Thody and Graham, 1998; Botchkareva *et al.,* 2001). The onset of these apoptotic events seems to be predetermined and finely orchestrated (Paus, 1998).

During catagen, the DP is transformed into a cluster of quiescent cells in close contact with the regressing HF epithelium, which moves from the subcutis to the dermis/subcutis boarder to contact the distal portion of the HF epithelium,

including the SHG (Botchkareva *et al.,* 2006). HF compartments involved in hair production are reduced in length by up to 70% during catagen. This allows the HF to regenerate in the next hair cycle after receiving the appropriate stimulation. Although catagen is often considered a regressive event, it is a delicately orchestrated, energy-requiring remodelling process, whose progression allows for renewal of a further generation of the HF. The close contact between DP and HF epithelium is essential for proper hair cycling. In the mice with *hairless* (*hr*) gene mutation, the disintegration of the DP and the HF epithelium during catagen leads to the loss of the capacity of the HF to re-enter anagen (Panteleyev *et al.,* 1999).

Apoptosis, a programmed cell death is indeed the most important characteristic feature of catagen, which is well-coordinated and occurs in the proximal part of the HF. Apoptosis is regulated differently in each HF compartment and distinct cell populations show different abilities to undergo apoptosis (Botchkareva *et al.,* 2006). The majority of the follicular epithelial cells and melanocytes are very susceptible to apoptosis, whereas DP fibroblasts, a population of keratinocytes and melanocytes selected for survival display a high resistance to apoptosis (Linder *et al.,* 1997; Botchkareva *et al.,* 2006).

During catagen, apoptosis may be considered as a wave, which begins from melanogenic area (late anagen) (Tobin *et al.,* 1999), and then later propagates to the hair matrix (early catagen) and then to the proximal/central outer and IRS's and hair shaft (mid-and late catagen) (Botchkarev and Paus, 2003). These apoptotic cells are phagocytised by macrophages and by neighbouring epithelial

cells, which fill the space left by apoptotic cells (“apoptotic force”) (Stenn and Paus, 1998).

Catagen may occur in HFs due to withdrawal of hair growth stimulators or signalling via death receptors. p53 is a transcription factor, identified as a possible mediator of apoptosis in the hair matrix keratinocytes during catagen. p53 knockout mice were shown to have reduced rates of catagen progression compared with control mice. Thus, p53 was identified as a pro-apoptotic protein in catagen (Botchkarev *et al.,* 2001b).

HFs also expresses distinct ‘death’ receptors during catagen stage of hair cycle including TNF receptor, Fas/CD95 and p75 NTR. This implicates involvement of ‘death’ receptors and their ligands in active remodelling of HF compartments during catagen.

TGF-β1 has been shown to promote premature catagen onset. The precise mechanism of TGF-β1 induced catagen is not know, however, it has been suggested that TGF-β1 may alter growth factors or their receptors, which promote cell proliferation and differentiation during late stages of anagen (Foitzik *et al.,* 2003). TGF-β1 has been shown to inhibit expression of HGF in human DP *in vitro* (Shimaoka *et al.,* 1994).

During catagen in mice, expression of the Bcl-2 to Bax ratio in hair matrix keratinocytes is markedly decreased compared with anagen levels (Lindner *et al.,* 1997; Botchkareva *et al.,* 2006). Also in catagen, the regressing HF has shown to express caspase 3, 4 and 7 (Lindner *et al.,* 1997; Botchkarev and

Paus, 2003), the enzymes, which participate in a cascade that is triggered in response to pro-apoptotic signals (Thornberry and Lazebnik, 1998).

The *hr* transcription factor has been shown to be an important regulator of apoptosis in the HF. Apoptosis is strongly increased in the hair matrix keratinocytes of *hr* mutant mice, resulting in premature entry of HFs to catagen from anagen. This subsequently leads to disintegration of the DP from the follicular epithelium and permanent hair loss (Panteleyev *et al.*, 1999). Vitamin D receptor (VDR) gene ablation in mice was shown to lead to hair loss and the phenotype resembled hair loss caused by mutations in the *hr* gene (Miller *et al.,* 2001), thereby suggesting a common signalling pathway for both *hr* and VDR. Subsequently it was shown that *hr* protein serves as a transcription co-repressor for the VDR (Hsieh *et al.*, 2003; Potter *et al.*, 2001). This suggests that interactions between *hr* protein and VDR may contribute to apoptosis in HF matrix keratinocytes (Botchkareva *et al.,* 2006).

The balance between proliferation and apoptosis during catagen can also be regulated by survivin (Botchkareva *et al.,* 2006). Survivin is a member of the apoptosis inhibitor protein (IAP) family, which has been identified as a possible regulator of cell proliferation as well as an inhibitor of apoptosis. Botchkareva and colleagues (2003) showed that survivin was expressed in the proliferating keratinocytes of the hair matrix and ORS of anagen HF but its expression disappeared with the onset of catagen. Pharmacological inhibition of survivin in the HF caused inhibition of HF length in organ culture model associated with

reduced levels of proliferative cell and increased TUNEL positive cells in HFs (Botchkareva *et al.,* 2003).

X-linked inhibitor of apoptosis protein (XIAP), which also belongs to the family of IAPs, is expressed in the hair matrix, ORS, and follicular papilla of early catagen HFs. During catagen, Eda A1 mRNA and Edar protein were shown to be expressed at high levels in the ORS and IRS. In *downless* mice lacking Edar, accelerated apoptosis in the ORS of the HF, along with decrease in expression of XIAP was found compared with control mice. It was suggested that Edar signalling is involved in regulating apoptosis in HF keratinocytes in part due to regulating XIAP expression in the HF epithelium during catagen (Fessing *et al.,* 2006).

The survival of the distinct HF cell populations during catagen is essential for the ability of HFs to regenerate in the next hair cycle. These cell populations include fibroblasts of the DP, epithelial stem cells and some of the daughter (transient amplifying) cells, and undifferentiated melanocytes that are able to restore the hair pigmentation unit in the next hair cycle. DP fibroblasts display very potent anti-apoptotic properties during the entire hair cycle. They are characterized by high levels of anti-apoptotic protein Bcl-2 and lack of the expression of apoptotic 'death' receptors (Lindner *et al.,* 1997).

1.13.4 Exogen

The process of hair shedding (exogen) in mice is now considered as a distinct stage of the hair cycle. Although the loss of club fibers occurs in the large majority of hairy mammals, very little is known about this cycle phase. This stage is the least studied aspects of HF activity. However, efforts have been made to understand and determine the significance of this stage. There are currently two theories on the process of hair shedding. The first is that hair shedding is a passive process. Passive process involves the removal of old hair shaft from previous HF cycle by mechanical force of the new growing hair shaft within the follicle (Milner *et al.,* 2002; Higgins *et al.,* 2009).

However, increasing evidence suggests that exogen is an active process. Studies from desmoglein-3 knockout mice and Bcl-x_L transgenic mice have shown increased club fiber retention in this stage. This demonstrated the importance and the involvement of adhesion molecules and programmed cell death in the retention and release of the club fiber in the epithelial sac (Koch *et al.,* 1998; Pena *et al.,* 1999). The importance of the exogen phase in the context of the HF biology is becoming more widely recognized, although more comprehensive investigation is required to understand the molecular mechanisms controlling this phase of the hair growth cycle (Higgins *et al.,* 2009).

1.14 The importance of Wnt signalling and its implications in the skin and hair follicle

1.14.1 Wnt/β-catenin signalling pathway

The Wnt signalling pathway has a crucial role in the HF development and HF cycling, tissue regeneration and in several other processes. Any mutations or deregulated expression of components of the Wnt pathway can lead to developmental defects and excessive levels β-catenin have been implicated in the development of several human cancers (Miller *et al.,* 1999; Klaus and Birchmeier, 2008).

At the core of the Wnt signalling pathway is β-catenin, a multi-functional protein with independent roles in cadherin-mediated cell adhesion and Wnt signal transduction (Willert and Nusse, 1998). The end result of Wnt/β-catenin pathway is the activation and free, signalling pool of β-catenin proteins in the cell that enters the nucleus and forms a complex with members of the Lef-1/TCF family of transcription factors to regulate expression of target genes. This can only be achieved by regulating the stability of β-catenin in the cell. In the absence of active Wnt signalling, β-catenin is degraded by initial phosphorylation and subsequent ubiquitination by the proteasome pathway, keeping the levels of free β-catenin low. In contrast, activation of the Wnt pathway prevents the phosphorylation and subsequent ubiquitination of β-catenin, which results in elevated levels of β-catenin in the cell (Miller *et al.,* 1999) (**Fig. 4**).

Phosphorylation and subsequent ubiquitination of β-catenin is a crucial process of regulating the levels of β-catenin as deregulation of β-catenin can lead to

several forms of cancer including colonal cancer (He *et al.,* 1998; Tetsu and McCormick, 1999), Familiar Adenomatous Polyposis (FAP) (Kinzler *et al.,* 1991; Nishisho, 1991), HF specific tumours such as, trichofolliculoma (Celso *et al.,* 2004) and pilomatricomas of the skin, and may contribute to hematological malignancies such as leukemia (Chan *et al.,* 1999; Jamieson *et al.,* 2004).

This crucial process of phosphorylation and subsequent ubiquitination of β-catenin is carried out by 'the destruction complex' consisting of four proteins: GSK-3, Axin, APC, and β-TrCP/Slimb. This elaborate complex of proteins is only formed when there is in-active Wnt signal. The function of this complex is to maintain low levels of β-catenin in the cell. Axin and APC proteins serve as scaffolds, binding both β-catenin and GSK-3 and facilitating the phosphorylation of β-catenin by GSK-3. Mutation or deletion of any of these proteins in the destruction complex have been shown to lead to highly stable forms of β-catenin that are constitutively active (Pai *et al.,* 1997; Yost *et al.,* 1996). These mutations are often found in a variety of human cancers (Miller *et al.,* 1999).

1.14.2 Wnt/β-Catenin signalling in skin and hair in health and disease

Several studies have suggested that multiple steps in skin and HF development/cycling are dependent upon a change in the transcriptional status of genes that are regulated by Wnt signalling pathway. Several Wnts and their Frizzled receptors have been shown to be expressed in the epidermis in a highly dynamic and complex pattern (Reddy *et al.,* 2001). In postnatal skin, two members of the Lef/TCF family are expressed. Tcf3 is expressed in the bulge and ORS of the HF, while Lef-1 is expressed in the ORS and matrix cells

(DasGupta and Fuchs, 1999; Merrill *et al.,* 2001). This suggests that Wnt/β-catenin signalling is important in HF cycling.

Subsequent studies in the skin have shown that selective ablation of Lef-1 gene in the epidermis inhibits HF development (van Genderen *et al.,* 1994). In contrast, transgenic mice overexpressing Lef1 resulted in *de novo* HF formation in the skin. Similarly, transgenic overexpression of a constitutively stable ('oncogenic') form of β-catenin induces additional HFs (Alonso and Fuchs, 2003). By contrast, conditional ablation of β-catenin blocks HF formation altogether (Huelsken *et al.,* 2001). In mice expressing stabilised β-catenin to the ligand-binding domain of a mutant oestrogen receptor (DeltaNβ-cateninER) induced resting HFs into anagen and promoted growth of existing follicles by topical application of synthetic 4-hydroxytamoxifen (4OHT) (Celso *et al.,* 2004). However, excessive activation of β-catenin has been shown to result in the formation of HF-derived tumours resembling trichofolliculomas. Pilomatricomas, which is an age related development of skin tumours in mice, have been shown to have higher levels of β-catenin characterized by dead hair cells surrounded by rapidly proliferating matrix cells (Gat *et al.,* 1998; Chan *et al.,* 1999). This establishes the canonical Wnt signalling as a crucial pathway required for HF induction, development (van Genderen *et al.,* 1994), and skin tumourigenesis (Chan *et al.,* 1999; Van Mater *et al.,* 2003; Celso *et al.,* 2004).

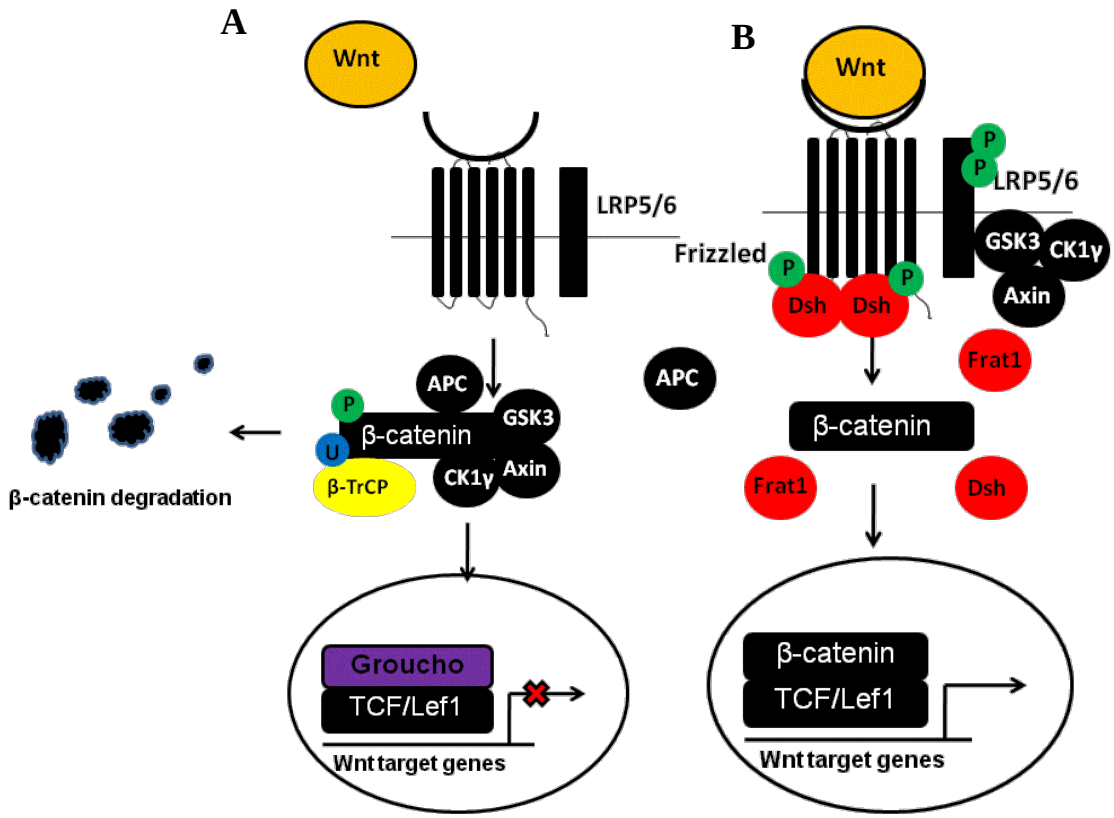


Figure 4. Schematic representation of the canonical Wnt signalling pathway. **A**) When Wnt receptor complexes are not bound by ligand, the serine/threonine kinases, CKI-γ and GSK3β, phosphorylate β-catenin, which is facilitated by Axin and APC. Phosphorylated β-catenin is recognized by β-TrCP complex, a component of a dedicated E3 ubiquitin ligase complex. This results in the ubiquitination of β-catenin, which is targeted for rapid destruction by the proteasome. In the nucleus, binding of Groucho to TCF/Lef1 transcription factors inhibits the transcription of Wnt target genes. **B**) In contrast, in the presence of Wnt signalling the Frizzled/LRP co-receptor complex activates the canonical signalling pathway. Dishevelled (Dsh) and GBP/Frat1 inhibit phosphorylation of β-catenin by inducing phosphorylation of LRPs by GSK3β and CK1γ instead, thereby regulating the binding of Axin. The binding of Axin away from the destruction complex results in the stabilization of β-catenin. In the nucleus, β-catenin displaces Groucho from TCF/Lef-1 transcription factors to promote the transcription of Wnt target genes. Abbreviations: TCF, T cell factor; Lef-1, lymphoid enhancing factor 1; CKI-γ, casein kinase I-γ; LRP, Lipoprotein receptor; U, ubiquitination; P, Phophorylated. (Adapted from Clevers, 2006).

1.15 The importance of BMP signalling in the skin and its appendages

Inductive processes during development and organogenesis are controlled by closely associated interactions between stimulators and inhibitors, the balance of which determines whether or not induction of HFs occurs. Epithelial-mesenchymal derived appendages, such as the HF, tooth, or feather involve interactions between hair placode keratinocytes and fibroblasts of underlying mesenchymal condensations (Hardy, 1992; Oro *et al.,* 1998; Philpott *et al.,* 1998) and include potent inhibitors of induction, some of which belong to the BMP family (Lamb *et al.,* 1993; Jung *et al.,* 1998; Botchkarev *et al.,* 1999). BMPs belong to a large group of secreted polypeptide growth factors that form the TGF-β superfamily. BMP signalling plays essential roles during embryonic development, postnatal tissue remodelling and regeneration in numerous organs by regulation of cell proliferation, differentiation, and apoptosis (Botchkarev and Paus 2003; Li, *et al.* 2003; Massague 2003; Mishina 2003; Plikus *et al.,* 2008). In the skin, BMP signalling has been shown to control crucial steps in HF development and cycling including inhibition of the initiation phase of the secondary HF morphogenesis (Botchkarev *et al.,* 1999), inhibitory influence on stem cell population of the HF during the resting phase of the hair cycle and in later stages it is required for proper control of keratinocyte differentiation into HF-specific cell lineages and hair pigmentation (Botchkarev *et al.,* 2001; Kobielak *et al.,* 2003; Andl *et al.,* 2004; Kin Ming *et al.,* 2004; Yuhki e*t al.,* 2004; Sharov *et al.,* 2005). Recent studies have also demonstrated a strong anti-tumour activity of the BMP signalling pathway in the skin epithelium (Blessing *et al.,* 1995; Zavadil *et al.,* 2007; Sharov *et al.,* 2009).

1.16 Skin Carcinogenesis

Skin cancer is one of the most common cancers in the world and rates of incidences continue to rise (Glick and Yuspa, 2005). During the last decade, there was a significant progress in the identification of molecular mechanisms underlying the development of the major cutaneous cancers, such as malignant melanoma, basal cell carcinoma, and squamous cell carcinoma (Li *et al.,* 2006; Owens and Watt, 2003). In particular, it was shown that mechanisms controlling skin and HF development seem to be very similar to carcinogenesis, and key signalling pathways such as Wnt/β-catenin signalling, Shh, Notch and TGF-β, which regulate skin and HF development are also implicated in the pathobiology of cutaneous neoplasias (Sharov *et al.,* 2009).

The chemically induced skin carcinogenesis in mice model has been an important tool for researches in investigating the molecular mechanisms contributing to human epithelial cancer development (DiGiovanni, 1992; Hennings *et al.,* 1993; Yuspa, 1994; Chan *et al.,* 2004, Abel *et al.,* 2009). Skin carcinogenesis in mice can be divided into three discrete steps: i) initiation, ii) promotion, and iii) malignant conversion (Hennings *et al.,* 1983). Initiation of tumour development in the skin involves an irreversible genetic change induced by a subcarcinogenic dose of a carcinogen such as 7,12-dimethylbenz(*a*)anthracene (DMBA). Initiation with DMBA leads to mutations in the *Ha-ras* gene (Yuspa, 1994), and, this stage usually does not involve morphological changes in skin. The promotion of tumour development requires the constant application of tumour promoters such as, 12-tetradecanoil-phorbol-13-acetate (TPA) to mouse skin. This leads to the tumour promotion, which is

characterized by a dramatic induction of epidermal cell proliferation and hyperplasia. The end result of the promotion stage is the development of premalignant clonal outgrowths known as papillomas (DiGiovanni, 1992; Yuspa, 1994; Chan *et al.,* 2004). For papillomas conversion into SCC, the accumulations of additional genetic changes are required (Hennings *et al.,* 1993; Chan *et al.,* 2004), for example, p53 mutations (Ruggeri *et al.,* 1991). p53 functions to induce growth arrest, promote apoptosis, block angiogenesis, or mediate DNA repair (Ko and Prives, 1996). The importance of p53 in preventing tumour formation was shown by the presence of mutations in the p53 pathway in nearly all cancers (Hollstein *et al.,* 1991), including in the skin (Caulin *et al.,* 2007).

1.17 MicroRNA- A New Layer of Gene Regulation

1.17.1 The Importance and Function of MicroRNAs

MicroRNAs (miRNA) are a large family of regulatory molecules found in all multi-cellular organisms. MiRNAs are small non-coding RNAs of approximately 22 nucleotides (nt) in length, which is created by enzymatic cleavage of endogenous primary transcript that contains a local hairpin structure (Bartel, 2004; Lee and Ambros, 2001; Ambros *et al.,* 2003; Gregory et al., 2004). Since the discovery of the first miRNA gene in *Caenorhabditis elegans* (*lin-4*) in 1993 (Lee *et al.,* 1993), approximately 800 miRNA genes have been identified in the human genome, which can exist either individually or form polycistronic clusters. Currently, it is estimated that miRNA genes make up about ~3% of the known genes and up to 30% of genes may be regulated by miRNAs in eukaryotes (Yu *et al.,* 2007). Of these 800 miRNAs, over 200 miRNAs have been discovered experimentally, and additional ones have been identified via computational approaches (Lagos-Quintana *et al.,* 2001; Lim, 2003; Lim *et al.,* 2003; Cheng and Li, 2008).

The function of miRNAs is just beginning to be understood, and it is evident that miRNAs have important regulatory roles in a variety of biological processes including control of developmental timing (Abbot *et al.,* 2005), growth control, cell differentiation (Yi *et al.,* 2008) and proliferation (Cheng *et al.,* 2005), embryonic stem cells (Murchison *et al.,* 2005) and in cancer development (Calin, 2004; Gregory and Shiekhattar, 2005). MicroRNAs regulate gene expression by base pairing with the 3' untranslated regions (3'UTRs) of target messenger RNAs (mRNAs), thus representing post-transcriptional level of regulation of gene

expression known as Post-transcriptional gene silencing (PTGS) (Lewis *et al.,* 2003; Cullen, 2004; Lewis *et al.,* 2005). MiRNAs and their mRNA targets appear to represent remarkably diverse regulatory networks as a single miRNA can bind to and regulate many different mRNA targets and/or conversely, several different miRNAs can bind to and cooperatively control a single mRNA target (Lewis *et al*, 2003). Thus, the discovery of miRNAs has added a new dimension to our understanding of complex gene regulatory networks.

1.17.2 Structure and Biogenesis of MicroRNA

MicroRNAs are transcribed by RNA polymerase II (Pol II) to generate a stem loop containing primary RNA transcript precursors known as pri-miRNA (Lee *et al.,* 2002), which can range from hundreds to thousands of kilobases in size (Lee *et al.,* 2004; Bushati and Cohen, 2007). Majority of genes encoding miRNAs are found within intergenic miRNA genes, while the rest of them are intragenic (Bohnsack *et al.,* 2002). Pri-miRNAs precursors are characterised by the presence of loop and stem structures with imperfect complimentary region. The mature miRNAs are possibly present on either side of the stem. Interestingly, these pri-miRNAs show a 5'cap structure (7-methylguanosine), are polyadenylated at the 3' end and have intronic and exonic regions similar to any other protein coding mRNA. Pri-miRNA is then processed into precursor-miRNA (pre-miRNA) by the nuclear RNase III enzyme Drosha (Kim, 2005a; Kim, 2005b; Bosjancic and Glavac, 2008). In the nucleus, Drosha interacts with essential co-factor DiGeorge Syndrome Critical Region Gene 8 (DGCR8) protein, (Pasha in Drosophila/DiGCR8), a protein containing two double stranded RNA-binding domains. The Droha-DGCR8 complex cleaves pri-miRNA, to form the ~70nt long containing 2nt 3'-overhang pre-miRNA (Denli *et al.,* 2004; Han *et al.,* 2004;

Chen, 2005; Gregory *et al.,* 2006; Yeom *et al.,* 2006). Knockdown of Drosha, using RNA interference (RNAi) technology, results in the strong accumulation of pri-miRNA, and the reduction of pre-miRNA and mature miRNA *in vivo* (Lee *et al.,* 2003), suggesting that Drosha is absolutely essential for miRNA biogenesis. It was shown that RNA stem-loops with a large, unstructured terminal loop (above 10nt) are the preferred substrates for the cleavage of Drosha (Zeng *et al.,* 2005). However, the precise mechanisms that the Drosha uses to discriminate miRNA precursors are still unknown.

It was shown that Drosha cleaves the pri-miRNA in an asymmetric manner near the base of the stem region and generates a double stranded 5'-phosphate and 3'-end two nucleotide overhang structure (Chendrimada and Sheikhatter, 2007). The efficiency of Drosha-DiGCR8 processing on pri-miRNA precursors depends on the terminal loop size, the stem structure and the flanking sequence of the Drosha cleavage site, because shortening of the terminal loop, disturbance of complementarity within the stem and removal or mutation of sequences that flank the Drosha cleavage site significantly decrease, if not abolishes, the Drosha processing of pri-miRNAs (Lee *et al.,* 2003; Zeng and Cullen, 2003).

The 2-nt 3'-end overhang, which is characteristics of RNases III endonucleases mediated cleavage, is recognised by Exportin-5 (Exp5). Exp5 is bound with guanosine triphosphate (GTP), a member of the Ran-dependent nuclear transport receptor family (Lund *et al.,* 2004). The depletion of nuclear GTP-bound Ran (RanGTP) and cellular Exp5 has shown to impair the export of pre-miRNA into the cytoplasm suggesting the crucial function of Exp5 in miRNA

biogenesis (Yi *et al.,* 2003; Lund *et al.,* 2004; Bohnsack *et al.,* 2004). On arrival, exportin-5-Ran/GTP is hydrolysed to exportin-5-Ran/GDP, releases pre-miRNAs into the cytoplasm. Pre-miRNAs are further processed in the cytoplasm by another RNase III enzyme known as Dicer (Bernstein *et al.,* 2001; Grishok *et al.,* 2001; Hutvagner *et al.,* 2001; Ketting *et al.,* 2001). Efficient Dicer cleavage also requires the presence of the 2nt 3'-overhang and a minimal stem length, suggesting a model in which the Dicer may recognize the end of the Drosha cleavage product, and therefore position the site of the second RNase-III cleavage on the stem of the miRNA precursors (Hutvagner *et al.,* 2001).

Dicer also associates with a double stranded binding protein, known as TRBP (the human immunodeficiency virus trans-activation response RNA-binding protein). This protein is required for Dicer processing of pre-miRNA in the cytoplasm. Studies investigating the role of Dicer-TRBP complex in miRNA biogenesis discovered that the knockdown of TRBP leads to Dicer destabilisation and consequentially loss of miRNA biogenesis (Chendrimada *et al.,* 2005). Dicer-TRBP complex cleaves the pre-miRNA and produces double stranded miRNAs ~22nt long. Differences in size may be a result of the presence of bulges and mismatches on the pri-miRNA stem. These miRNAs are indistinguishable from a small-interfering RNA (siRNA), the mediator of RNAi. Cleavage of pre-miRNA produces short-lived miRNA duplex, where one strand is the duplex is degraded, while the other mature single stranded miRNA is then incorporated into an RNA Induced Silencing Complex (RISC) (Bartel, 2004). It is not yet clear how the separation of the double stranded miRNA duplex occurs and how the mature strand is incorporated into the RISC complex.

Studies have suggested that separation of strands may be due to the low stability of the 5'-end of the mature strand and thus facilitate the mature/passenger strand separation. Other suggestions include: (i) the role of an unidentified helicase, which unwinds the duplex. The separated mature miRNA strand is then incorporated into the RISC, (ii) the double stranded miRNA duplex may be inserted into the RISC and the RISC then recognises and cleaves the passenger strand as a substrate, leaving the mature strand to bind to target mRNA (Bartel, 2004; Chendrimada and Sheikhatter, 2007). In addition, it has been postulated that TRBP is required for the recruitment of Dicer processed double stranded miRNA into the RISC complex for mature/passenger strand separation (Chendrimada *et al.,* 2005).

Comparable to Drosha, Dicer also contains two tandem RNase-III domains and also produces pre-miRNA by a single cleavage event. Currently, given the lack of knowledge regarding the exact structural elements that guide Dicer and Drosha cleavage, it is complicated to speculate about their precise biochemical mechanisms. However, it is highly possible that Drosha and Dicer share closely related mechanisms for the processing of miRNAs (Kim, 2005a).

1.17.3 Post-transcriptional Regulation by MicroRNAs

Even though, all miRNA-mRNA interactions appear to downregulate gene expression post-transcriptionally, the level of regulation of target mRNA by miRNAs has been shown to vary substantially, from less than two-fold to greater than ten-fold, depending on the specific miRNA-mRNA target combinations. Although the crucial factors that define the different magnitudes of regulation remain unknown, mRNAs that are only modestly regulated must be translated. Whether the level of translation of target mRNAs is determined by the accessibility of the mRNA to miRNAs or by other factors is yet to be defined (Aukerman and Sakai, 2003; Maroney *et al.,* 2006; Jackson and Standart, 2007).

MiRNAs are believed to regulate gene expression post-transcriptionally by forming Watson-Crick base pairs between miRNA recognition element (MRE) in target mRNAs 3' UTR and 5'-region of miRNA, called the seed region (Brennecke *et al.,* 2005). In animals, most miRNAs are believed to base pair imperfectly with their target mRNA(s) and these interaction sites are enriched in 3'-UTRs (Bartel, 2004). The amount of complementation between the miRNA and its target mRNA determines the fate of the bound target mRNA (Hutvagner and Zamore, 2002; Cheng and Li, 2008). Perfect pairing causes the target mRNA to be cleaved, as is the case in the majority of plant miRNAs (Dugas *et al.,* 2004). Imperfect base pairing alternatively leads to the blockade of translation, as seen in the majority of animal miRNAs (**Fig. 5**) (Wightman *et al.,* 1993; Reinhart *et al.,* 2000).

One of the first examples of the role of endogenously expressed miRNAs in down-regulating gene expression was *lin-4*, which negatively regulates its target, *lin-14*, by repressing its translation (Olsen and Ambros, 1999). Base pairing between *lin-4* and *lin-14* has been shown to be vital for their interaction *in vivo*, as mutations that affect their base pairing complementarity compromises or abolish this negative regulation (Lee *et al.,* 2002). These genetic interactions between *lin-4* and *lin-14* stimulated a series of molecular and biochemical studies demonstrating that the direct, but imprecise, base pairing between *lin-4* and the *lin-14* 3′ UTR was crucial for the ability of *lin-4* to control *lin-14* expression via the regulation of protein synthesis (Lee *et al.,* 1993; Olsen and Ambros, 1999; Kim, 2005a).

1.17.4 RNA Inducing Silencing Complex (RISC)

Even though, currently the progress in identifying protein and RNA components of the RISC has advanced considerably, the biochemical mechanism by which this complex functions still remains largely unknown. Genetic screens combined with biochemical purification have shown that the RISC is composed of Piwi-Argonaute-Zwille (PAZ) complex (Hammand *et al.,* 2001). Dicer cleavage products are incorporated into PAZ-containing RISCs, which mediates mRNA degradation or translational suppression (Hannon, 2002; McManus and Sharp, 2002; Tahbaz *et al.,* 2004). Current structural analysis of PAZ complex has shown that it is able to interact at low-affinity with the 3′ end of single stranded RNAs (Bernstein and Caudy, 2001; Grishok *et al.,* 2001; Zeng and Cullen, 2004). This alliance also permits the PAZ domain to interact with double stranded RNAs that present 2-nt 3′ end overhangs, such as those that result from Drosha cleavage (Bohnsack *et al.,* 2004; Grishok *et al.,* 2001; Zeng and Cullen, 2004).

Ago2 is the catalytic domain of the PAZ complex required for efficient cleavage of target mRNA. Ago2 is a member of the Argonaute protein family and the only member in humans that is associated with both siRNA and miRNA silencing pathways. It was shown that mutations within the Ago2 inactivate RISC, suggesting the importance of Ago2 in miRNA-induced mRNA silencing (Liu et al., 2004; Rand *et al.,* 2005).

Early analyses of miRNA involvement in regulation of gene expression did not show changes in the target mRNA levels, whereas the amounts of proteins encoded by those mRNAs were significantly reduced. In addition, miRNAs targeted mRNAs were found on polysomes, suggesting that the block to translation occurred after initiation of protein synthesis. These studies have suggested that RISC containing miRNA (miRNP complex) may have direct effects on target mRNA by inhibition of initiation of translation, which results in prevention of ribosome association with the target mRNA (Olsen *et al.,* 1999).

Additional mechanism of regulation of miRNA may involve post-initiation repression of target mRNAs. This includes premature 'ribosome drop off', slowed or stalled elongation, and co-translational protein degradation. In addition to direct effects on translation, miRNPs can have effects on targeted mRNAs by promoting deadenylation (Giraldez *et al.,* 2006), which might result in degradation of target mRNA. Both de-adenylation and degradation of target mRNA may take place in cytoplasmic P bodies, which are enriched with factors involved in mRNA degradation. It is possible that miRNA-targeted mRNAs could

be sequestered from the translational machinery and degraded or stored for subsequent use (Nilsen, 2007).

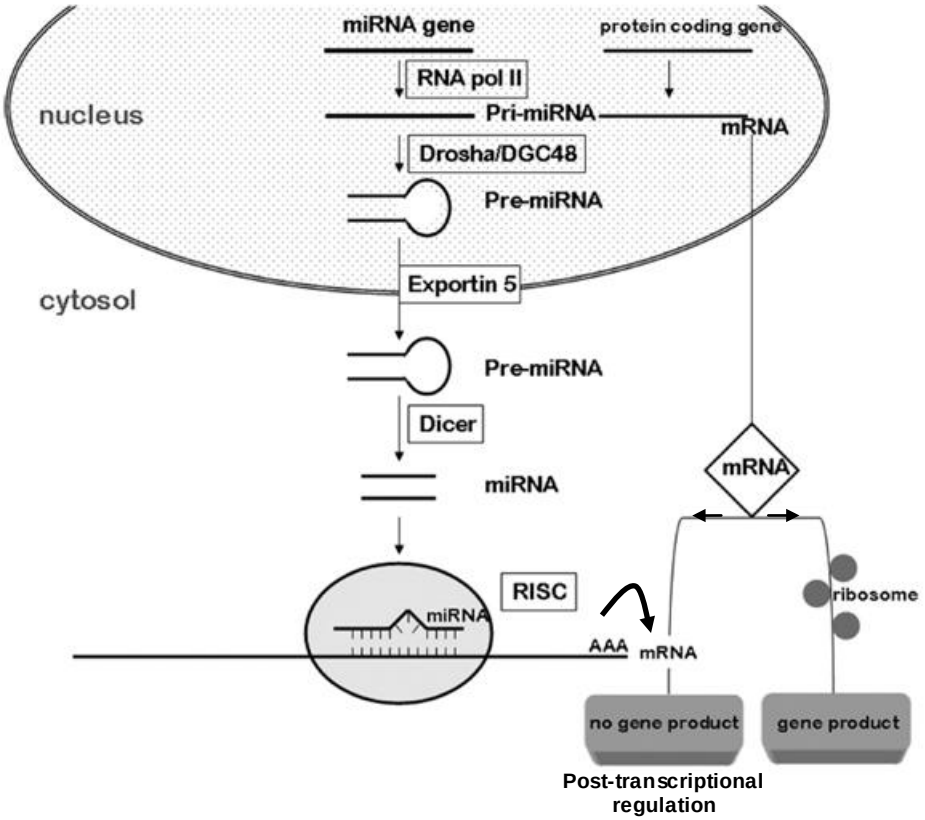


Figure 5: MicroRNA Biogenesis. Primary microRNA (pri-miRNA) is synthesized in the nucleus by RNA polymerase II (RNA Pol II). The RNA endonucleases Drosha and its cofactor DiGCR8 cleave pri-miRNA to produce precursor miRNA that is about 70 nucleotides long. This product is exported to the cytoplasm by Exportin 5 where it is cleaved again by the second RNA endonucleases Dicer to form the approximately 22nt-long mature miRNA. One of the double-stranded miRNA is incorporated to the RNA-induced silencing complex, where by imperfect base matching with the 3'UTR and the seed region of miRNA, it captures the target mRNA in the complex. This causes inhibition of translation by the ribosome. If perfect base pairing with the 3' UTR and the seed region of miRNA, it captures the target mRNA in the complex resulting in mRNA degradation. Abbreviations: Pre-miRNA, Precursor microRNA; RISC, RNA induced silencing complex; mRNA, messenger RNA; DGCR8, DiGeorge Syndrome Critical Region Gene 8 (Adapted from Shilo *et al.,* 2007).

1.17.5 MicroRNA-mediated control in the Skin and Hair Follicle

We are currently in the early stages of miRNA research and the role of these miRNAs in the body's largest organ, the skin, is currently being intensely investigated. Recent significant findings about the possible roles of miRNAs in skin and HF development, which were uncovered initially by Andl and colleagues (2006) and Yi and colleagues (2006), may provide important insights that lays the foundation for research in areas closely associated with cutaneous biology, including wound healing, skin ageing, skin carcinogenesis, angiogenesis research, and HF development and cycling.

Pioneering work initially carried out by Andl and colleagues (2006) and subsequently Yi and colleagues (2006) attempted to determine whether Dicer is essential for the development of HFs and/or epidermis. Dicer is one of the key enzymes in the processing of miRNA to functional mature miRNA. Ablation of Dicer arrests mechanisms triggered by mature miRNA. It was first discovered by *in situ* hybridization of mouse embryos and mouse literates that Dicer is present in both epidermis and HF ORS. Keratinocyte-specific deletion of the Dicer gene was carried out in mice. Dicer mutant mice were at first indistinguishable from control mice group. However, by postnatal day 7, mice were characterised by stunted HFs, which lacked external hair growth. Evagination of the epidermis by HFs and replacement of HFs by cyst-like structures of epithelial cells within the dermis was observed in Dicer mutant mice. In addition, expression of the progenitor cell marker Keratin 15 was absent in the skin of newborn Dicer mutant (Andl *et al.,* 2006; Yi *et al.,* 2006).

In contrast to decreased proliferation in the HF, Dicer mutant mice showed hyperproliferative epidermis, with abnormal expression of basal and suprabasal specific keratins (i.e, Krt14) compared with control mice. Dicer mutant mice also showed reduced expression of Shh and *Notch1*, which were previously identified as important regulators of HF development. Shh was shown to regulate follicular proliferation and down growth of HFs. *Notch1*, regulates the maintenance of IRS precursors and epidermal proliferation (Nicolas *et al.,* 2003; Pan *et al.,* 2004) and inactivation of *Notch1* in the skin was shown to lead to almost complete hair loss followed by cyst formation (Vauclair *et al.,* 2005).

To identify HF-specific miRNAs, global analysis of miRNAs expression was performed in transgenic mice overexpressing Dkk1, a potent inhibitor of the Wnt signalling pathway. Dkk1 transgenic mice are characterised by altered HF development (Andl *et al.,* 2006), therefore this model was chosen for the identification of miRNAs, which could possibly be involved in the control of HF development. Expression of several miRNAs, in particular, miR-200b and miR-196a, have emerged in the skin of Dkk1 transgenic mice suggesting their importance for HF development (Andl *et al.,* 2006).

Research work carried out by Yi and colleagues (2006) further explored the role of miRNA in mouse skin during HF development. They discovered that many miRNAs are differentially expressed by epidermal and HF keratinocytes. These findings support the current belief that target mRNAs in the skin are efficiently regulated by miRNAs (Andl *et al.,* 2006; Yi *et al.,* 2006).

Following studies of Dicer knockout mice, Yi and colleagues (2009) knocked out the essential co-factor DGCR8 of the miRNA processing machinery. Similar to Dicer-deficient mice, DGCR8 knockout animals showed elevated apoptosis in skin epithelium and evaginated HFs. Severe defects in epidermal differentiation and barrier formation leading to dehydration and neonatal death (Yi *et al.,* 2009). These studies demonstrate that miRNAs play a critical role in skin morphogenesis and HF development.

Recently, miR-203 was identified as a keratinocyte-specific miRNA in the skin (Yi *et al.,* 2008; Lena *et al.,* 2008). These studies showed miR-203 by targeting p63 restricts epidermal proliferation and induces epidermal differentiation and therefore, miR-203 defines a molecular boundary between proliferation and differentiation in epidermal suprabasal cells.

1.17.6 MicroRNAs and skin disorders

1.17.6.1 MiRNAs and skin cancer

Many miRNAs have been implicated in targeting mRNAs, which are involved in cell proliferation, differentiation and apoptosis (Bartel *et al.,* 2004; Gregory and Shiekhattar, 2005). These biological processes are commonly altered during the development of skin disorders including skin cancer. To identify a potential role of global miRNAs in skin tumourigenesis, Kumar and colleagues (2007), inhibited maturation of all miRNAs by targeting three essential miRNA processing regulators: the two endonucleases (Dicer and Dorsha) and Drosha processing partner DiGCR8 in cancer cell lines. They found that abrogation of global miRNA processing machinery leads to transformed cell phenotype and more invasive tumours compared with controls (Kumar *et al.,* 2007). This study has further enhanced our understanding of the global impact of elimination of miRNAs and its possible anti-invasive and anti-metastasis abilities in several cancer cell lines. However, the role of miRNAs in skin disorders and skin tumourigenesis is still in its infancy (Kumar *et al.,* 2007). Particularly, a role of individual miRNAs in skin pathobiology remains unknown.

MiRNA-21 is one of the most upregulated miRNAs in a number of cancer profiling experiments and has been shown to be upregulated in majority of cancer cell lines tested to date (Tong and Nemunaitis, 2008). In the skin, miR-21 has been shown to be also upregulated in some inflammatory skin disorders such as psoriasis and atopic eczema (Sonkoly *et al.,* 2007; Friedland, *et al.,* 2009; Zibert *et al.,* 2010). Furthermore, miR-21 was shown to be overexpressed in several tumours, including melanoma (Lu *et al.,* 2008) and has been implicated in squamous cell carcinoma (SCC). SCC is an extremely common

skin cancer (Glick *et al.,* 2005) and even though increased understanding and improved prevention strategies are currently being employed, SCC still continues to rise worldwide. Dziunycz and colleagues (2010) showed elevated expression of miR-21 in SSC suggesting that miR-21 may regulate SCC formation, contribute to development and/or progression of SCC (Dziunycz *et al.,* 2010).

Recently, miR-21 has been identified as a crucial functional target of several anti-tumourigenic pathways. For example, BMP-6 exerts its anti-metastasis effect by a mechanism involving transcriptional repression of miR-21 in breast cancer (Du *et al.,* 2009). MiR-21 regulation was demonstrated in response to BMP signalling initially during normal development and homeostasis of the vasculature (Davis *et al.,* 2008). In addition, miR-21 has been identified as a direct regulator of BMPRII expression and deregulation of BMPRII, leads to prostate cancer progression (Ye *et al.,* 2007; Qin *et al.,* 2009).

1.17.6.1 MiRNAs and skin inflammatory diseases

Psoriasis is a common chronic inflammatory skin condition characterized by accelerated keratinocyte proliferation, reduced apoptosis and epidermal infiltration of inflammatory cells that leads to the formation of skin plaques. TNF-α is a pro-inflammatory cytokine shown to play an important role in the pathogenesis of psoriasis (Ettehadi *et al.,* 1994). Recent studies have shown a possible involvement of miRNAs in the pathogenesis of chronic inflammatory skin diseases such as psoriasis and atopic eczema. Three miRNAs have been implicated so far in these skin disorders, including: miR-203, miR-146a and miR-125b. MiR-203 was the first miRNA to be implicated in the pathogenesis of

psoriasis by regulating inflammation, proliferation and morphogenesis-associated processes in the skin (Chen *et al.,* 2004; Sonkoly *et al.,* 2007). Activator of transcription 3 (STAT3) has been shown to be constitutively activated in epidermal keratinocytes of human psoriatic lesions (Sano *et al.,* 2005). Following genome-wide analysis of miRNA expression, upregulation of miR-203 was shown to lead to downregulation of suppressor of cytokine signalling-3 (SOCS-3) expression in psoriatic skin. SOCS-3 is an inhibitor of the signal transducer and STAT3 pathways (Sonkoly *et al.,* 2008). Therefore, targeting of miR-203 may lead to inhibition of STAT3 and potentially improving clinical prognoses in psoriatic patients (Sano *et al.,* 2008).

In addition to miR-203, miR-146a has also been shown to be overexpressed in many psoriatic skin lesions (Sonkoly *et al.,* 2008). MiR-146a was shown to target the TNF receptor-associated factor 6 (TRAF6) and IL-1R-associated kinase (IRAK), all of which are all involved in the TNF-α pathway, which contributes to psoriatic skin inflammation. It was discovered the expression of miR-146a was NFkappaβ-dependent (Taganov *et al.,* 2006). In addition, it was shown that NFkappaβ activation resulted in the inhibition of TNF-α-induced apoptosis, which may be regulated by miR146a overexpression, potentially contributing to the pathogenesis of psoriasis (Tang *et al.,* 2001). MiR-125b expression has been shown to be down-regulated in psoriasis (Sonkoly *et al.,* 2008). MiR-125b is involved in post-transcriptional repression of TNF-α. MiR-125b downregulation reduced inhibitory effect on TNF-α (Sand *et al.,* 2009). Furthermore, several other miRNAs have been implicated in human melanoma (Philippidou *et al.,* 2010) and atopic dermatitis (Sonkoly *et al.,* 2010). Recent global microarray

profiling revealed that miR-155 was upregulated in human melanoma and atopic dermatitis compared with relatively low expression detected in normal skin (Philippidou *et al.,* 2010; Sonkoly *et al.,* 2010).

Taken together, miRNAs are important players involved in the control of skin and HF development and deregulation of the expression of miRNAs in skin leads to the development of skin and HF disorders.

Aims

HF development and growth involves all the important physiological processes found in the human body. These include interactions between different cell types, proliferation, migration, controlled cell growth, cell differentiation and cell death. As a consequence, the importance and value of the HF as a model for scientific research is way beyond its scope for cutaneous biology or dermatology alone (Tobin, 2005).

MiRNA research in the area of dermatology is relatively new. MiRNAs have been investigated intensely in dermatological research areas recently, leading to many intriguing discoveries (Yi and Fuchs, 2009). The elucidation of the molecular mechanisms that regulate skin and HFs homeostasis may provide new insights into the management and effective treatment of several skin and HF disorders and cancers.

This project investigates the role of miRNAs using HF as a model and examines the expression levels and location of certain miRNA during HF morphogenesis, HF growth/cycling, and tumourigenesis in murine skin. This will be relevant to researchers investigating the fundamentals principles of organogenesis, oncogenesis, growth and regeneration of skin and HFs.

Therefore, the aims of this study were i) to define changes in the miRNA profiles in skin during hair cycle-associated tissue remodelling, ii) to determine the role of individual miRNAs in regulating gene expression programs that drive HF growth,

involution and quiescence, iii) and to explore the role of miRNAs in mediating the effects of BMP signalling in the skin.

Chapter II

MATERIALS AND METHODS

2.0 Materials and Methods

2.1 Animals

All animal work was performed under the project license of Professor Vladimir Botchkarev and carried out by Dr Andrei Mardaryev (University of Bradford, UK). Mice had free access to food (standard rodent diet) and tap water. The animal room was maintained under continuous 12-hour light and dark cycles with temperature and humidity at 21±1^{o}C and 40-60%, respectively.

C57BL/6 mouse model was used for all animal experiments, under several different experimental conditions. Each control and experimental groups contained at least five mice per treatment/time point, which were harvested according to accepted practises (Paus *et al.,* 1999). Skin samples were collected from mice at postnatal days 12-23 (P12-P23). To induce hair cycle, depilation of the back skin was performed by applying very briefly a hot wax/rosin mixture on the dorsal skin of 7-week old mice followed by plucking of hair shafts by wax/rosin mixture, which induces homogeneous anagen development over the entire depilated back of the mouse. After the induction of full anagen in depilated back skin, the consecutive stages (catagen and telogen) spontaneously occur, in a fairly homogeneous manner (Muller-Rover, *et al.,* 2001). In depilated induced cycle: skin were harvested at the telogen stage of the hair cycle (unmanipulated skin), as well as at anagen II [3 days post depilation (dpd)], anagen IV (5 dpd), anagen VI (8-12dpd), and catagen (16,19 dpd), using ≥5 mice/time point.

Harvested skins were covered in embedding OCT-mounting medium (Tissue-Tek®, CA USA) and then frozen by adding to liquid nitrogen (Paus *et al.,* 1999).

Transgenic mice overexpressing BMP antagonist *noggin* (under K14 promoter) were generated on FVB background by a construct containing mouse *noggin* cDNA (kindly provided by R. Harland) and human growth hormone poly-A sequence as it was described previously (Sharov *et al.,* 2009).

2.1.1 Chemically induced skin carcinogenesis in mouse skin:

The mouse skin model represents one of the best-established *in vivo* models for the study of the sequential and stepwise development of tumours. Scientists have been able to use this model for multi-stage chemical skin carcinogenesis to evaluate both novel skin cancer prevention strategies, and genetic manipulation on tumour initiation, promotion and progression in the skin (DiGiovanni, 1992; Abel *et al.,* 2009). Here we have used a two-stage protocol for mouse skin tumourigenesis. FVB 8-week-old mice were exposed to a single dose of subcarcinogenic of DMBA (250µg/ml) (Sigma, Sigma, St. Louis, MO, USA), followed by the applications of TPA (40µg/ml) (Sigma, Sigma, St. Louis, MO, USA) twice a week for up to 15 weeks. FVB mice were chosen as these mice were shown to be more sensitive to chemical induction of SCC of the skin (Hennings *et al.,* 1993). Skins were collected at day 0 (untreated), 15 and 28 weeks after the beginning of the experiment.

2.1.2 Pharmacological experiments and morphometric analyses:

Synthetic miR-31 inhibitor (anti-miR-31) or inhibitor negative control (Dharmacon Inc, USA) was administered subcutaneously to back skin of 8-week-old C57BL/6 mice in concentration 20 µM using AteloGene kit (Koken Co, Japan), a system which provides local sustained, slow-releasing oligonucleotide delivery (Minakuchi,*et al.,* 2004;Takeshita *et al.,* 2005). In the first experiment, anti-miR-31 treatment was performed on 1, 2, 3, and 4 dpd (days of post depilation), and skin samples were collected on 5 dpd. In the second experiment, anti-miR-31 was administrated on 4, 5, 6, and 7 dpd, and skin was harvested on 8 dpd. In each experiment, ≥4 or 5 mice/time point were used for analyses in both experimental and control groups. Collected samples were processed for morphometric, histological, and biochemical analyses. In control and anti-miR-31 treated skin, HF morphology and staging was carried out by examining at least 50 different HFs per mouse. Alkaline phophatase detection method was used, because it allows precisely the identification of the defined stages of HF cycling by staining the DP (Handjiski *et al.,* 1994).

2.2 Cell culture

2.2.1 Culturing of Primary mouse epidermal keratinocytes:

Primary mouse epidermal keratinocytes (PMEKs) were prepared from newborn mice at P1-3, as described elsewhere (Lichti *et al.,* 2008). PMEKs were grown in Eagle's minimum essential medium (EMEM) calcium-free medium (Lonza, Slough, UK) supplemented with 0.05mM calcium, at 33°C, 8% CO_2 (Scientific Laboratory Suppliers, Hessle, UK) until 60–70% confluent.

2.2.2 Culturing of HaCaT cells:

HaCaT cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Lonza, Slough, UK) supplemented with heat-inactivated 10% FBS (Sigma, St. Louis, MO, USA), 5% L-glutamine (Sigma, St. Louis, MO, USA) and 10mg/ml of antibiotics (Sigma, St. Louis, MO, USA) at 37°C, 5% CO_2 (Scientific Laboratory Suppliers, Hessle, UK) until 60–70% confluent.

2.2.3 Experimental transfection and treatment of primary mouse epidermal keratinocytes:

To define the effects of the loss or gain of selected miRNAs activity on the keratinocytes, PMEKs were transfected with 200nM synthetic miR-31 inhibitor (anti-miR-31), miR-31 mimic (pro-miR-31), miR-21 mimic (pro-miR-21), miR-214 mimic (pro-miR-214) or miRNA negative controls (Dharmacon Inc, USA), respectively, using Lipofectamine RNAiMax (Invitrogen, UK). Lipofectamine RNAiMAX and synthetic miRNAs mixture were incubated for 20mins at room temperature in a ratio of 2:1 and subsequently added to PMEKs and incubated for 4 hours at 33°C, 8% CO_2. Cells were harvested 24 hours after transfection and used for further analyses.

2.2.3.1 Lithium chloride (LiCl) treatment of keratinocytes:

To activate Wnt signalling in the keratinocytes, 10mM LiCl was added for 2 hours (Klein and Melton, 1996), followed by transfection of cells with 200nM pro-miR-214 as described above (step 2.2.3) for 4 hours at 33°C, 8% CO_2. Cells were then harvested 24 hours after transfection.

2.2.3.2 BMP4 treatment of keratinocytes:

PMEKs were treated with 100ng/ml of BMP4 (R&D system, UK) for 4 and 12 hours and collected for further analysis as described previously (Fessing *et al.,* 2010). To explore the regulatory effects of miR-21 on BMP-induced gene expression, PMEKs were transfected with 200nM synthetic pro-miR-21 or miRNA negative controls as described above (step 2.2.3) for 4 hours. Next, after quick wash in PBS, cells were treated with 100ng/ml of BMP4 for 4 hours at 33°C, 8% CO_2. This was followed by their harvesting for total RNA isolation

2.3 RNA Isolation and Microarray analysis

2.3.1 Isolation from the skin:

Total RNA was isolated from snap frozen experimental samples by using miRNeasy kit (Qiagen, Crawley, UK). One ml of Qiazol RNA isolation reagent (Sigma, St Louis, MO, USA) was added to 50mg of tissue for lysis, and RNA was isolated following manufacturer's instructions. For miRNA microarray analysis in the skin, 5µg of RNA was used. Total RNA was isolated from the skin of neonatal mice at postnatal days 12-15 (P12-P15), i.e. when HFs complete morphogenesis and actively produce hair (anagen-like stage), as well as at P16-P17 and P20-P23 (i.e., during catagen and telogen stages, respectively) (Paus *et al.,* 1999). MiRNA microarray analysis was performed by LC Sciences, (Houstan, Texas, USA).

2.3.2 Isolation from cultured keratinocytes:

Total RNA was isolated from PMEKs by using miRNeasy kit. One ml of Qiazol RNA isolation reagent was added to 1 x10^7cells for lyses, and RNA was isolated following manufacturer's instructions. MiRNA microarray analysis was performed by LC Sciences. mRNA microarray analysis was performed by Mogene Co. (St. Louis, MO, USA) using 41K Whole Mouse Genome 60-mer oligo-microarray (manufactured by Agilent Technologies).

2.4 Real Time PCR

2.4.1 Principles

Real-time PCR allows the possibility of detecting and measuring the amplified product as the reaction is progressing, that is in "Real Time". This is in contrast to conventional PCR, where the amplified product is only possibly detected by an end-point analysis by running amplified DNA on an agarose gel, until after the reaction has finished. There are a number of fluorescence molecules that can be used for real time PCR to measure DNA product levels. These include: DNA-binding dyes such as, SYBR GREEN® and fluorescently labelled sequence specific primers, TaqMan Assay®. Each Real-Time PCR machine is equipped with specialized thermal cyclers with fluorescence detection modules, which are capable of monitoring the fluorescence signal as amplification occurs. The measured fluorescence is proportional to the amount of amplified product in each cycle. At first, the fluorescence signal is not detected even though the product of interest is accumulating exponentially but eventually enough amplified product will be produced to produce a detectable signal. This is known as threshold cycle

(C_t). C_t is measured during the exponential phase and is determined by the amount of template at the start of the amplification phase (McPherson and Møller, 2006). C_t was used to determine the relative expression of target genes in experimental samples.

2.5 Analysis of miRNA expression

2.5.1TaqMan Real Time PCR

2.5.1.1 Principle:

cDNA synthesis of miRNA requires a specialised novel stem loop Reverse Transcription (RT) primer during reverse transcriptase reaction. This stem loop RT primer overcomes the fundamental problem of miRNA being very short (approx. 22nt long). The TaqMan® Assay kit (Applied Biosystems, Foster city, CA, USA) overcomes this problem by extending the 3' end of the mature miRNA, via binding of RT primer, therefore, providing a longer reverse transcription product suitable for real time PCR (Chen and Guegler, 2007; Schmittgen *et al.,* 2008).

2.5.1.2 TaqMan complementary DNA (cDNA) Synthesis:

RNA was isolated from cells or tissue samples using Qiazol lysis reagent (Qiagen, Crawley, UK) as described above (step 2.3) followed by DNase I treatment and purification on RNeasy columns, as following the manufacturer's instructions. cDNA was synthesised using TaqMan® Assay Kit (Applied Biosystems, Foster City, CA, USA) following manufacturer's instructions. The following master mix was made up for each reverse transcription reaction:

100mM of dNTPs, 1µl of multiscribe reverse transcriptase, 1.5µl 10x reverse transcriptase buffer, 0.19µl RNase inhibitor (20units/µl) and 4.6µl water. 7µl of this master mix was combined with 5ng of total RNA. This was mixed gently and 3µl of RT primer (Applied Biosystems, Foster City, CA, USA) assay was added to reaction mix followed by brief centrifugation. cDNA was synthesised using a Master Cycler gradient machine (Eppendorph, Cambridge, UK) under the following cycling conditions: 16°C for 30 minutes, followed by 42°C for 30 minutes and 85°C for 5 minutes.

2.5.1.3 TaqMan real-time PCR procedure for detection miRNA expression:

Real-time PCR was performed using TaqMan® Real Time PCR Assay kit (Applied Biosystems, Foster City, CA, USA) and MyiQ single-colour real-time PCR detection system (Bio-Rad Corp, UK). Each TaqMan® qRT-PCR consisted of 1µl TaqMan Real Time PCR Assay (Applied Biosystems, Foster City, CA, USA), 10µl TaqMan 2X Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 1.33µl of cDNA template and 7.67µl of water. Real-time PCR was performed under the following cycling conditions: 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 minute. QRT-PCR was performed in triplicates. Differences between samples and controls were calculated using the Genex database software (Bio-Rad Corp, UK) based on the Ct ($^{\Delta\Delta}C_t$) Livak equitation method and normalized to the corresponding Small nucleolar RNA 202 values (SnoRNA). SnoRNAs are constitutively expressed across all tissues; the use of same assay chemistry and RNA isolation protocols makes snoRNAs ideal candidates for normalization of miRNA expression (Chen

and Guegler, 2007). Data were pooled, mean ± SEM was calculated, and statistical analysis was performed using unpaired Student's t test.

2.6 Analysis of mRNA expression

2.6.1 First-strand qRT-PCR:

RNA was isolated from the cells or tissue as described above (step 2.3). For RT-PCR, 1µg of each RNA sample was used as a template for cDNA synthesis using Superscript III first-strand synthesis system (Invitrogen, Paisley, UK), 50ng of random hexamers and 1µl of water. The mixture was incubated in a thermal cycler at 65°C for 5 minutes, and then immediately place on ice for at least 1 minute. The following was added to the mixture (on ice): 10µl of 2X First-Strand Reaction Mix and then 2µl of Superscript™ III/RNaseOUT™ Enzyme Mix. The following cycling conditions were used: 25°C for 10mins, followed by 50°C for 50 minutes, and termination at 85°C for minutes.

2.6.2 Real-Time PCR:

QRT-PCR was performed with iQ SYBR Green Supermix (Bio-Rad Corp, UK), using 10ng cDNA and 1uM primers. PCR primers were designed with Beacon Designer software (Premier Biosoft International; **Table 1**). Amplification was performed at the following conditions: 95°C for 5 min, followed by 40 cycles of denaturation (95^{o}C for 15 sec), annealing (30 sec at temperature experimentally determined for each primer pairs) and elongation (72^{o} C for 15 sec). For each gene of interest, qRT-PCR was performed in triplicates. Differences between samples and controls were calculated using the Genex database software (Bio-

Rad Corp, UK) based on the Ct ($^{\Delta\Delta}C_t$) equitation method and normalized to the corresponding Glyceraldehyde 3 phosphate dehydrogenase (GAPDH). Data analysis was performed as described above. Data were pooled, mean ± SEM was calculated, and statistical analysis was performed using unpaired Student's t test.

Table 1: List of primer sequences for qRT-PCR

Accession Number	Sequence Definition	Sense/Anti-sense Primers
NM_015732	Axin2	CACCTCTCCTCTGTTACCTTC GTCAACGCTCTGCCCTAC
NM_026505	BMP and activin membrane-bound inhibitor (BAMBI)	TCCTGTATCTGTTTCCTTCCTGAG ACTGATGGTGGTGACTGTGTAG
NM_010055	Distal-less homeobox 3 (Dlx3)	CCAAATCCACTCCTCTCTG GTCTTGCCTGGTCTATCTC
NM_001165902	Catenin (cadherin associated protein), beta 1 (Ctnnb1)	GCCACCAAACAGATACATAC CCTCTCAGCAACTCTACAG
NM_0076331	CCND1 (Cyclin D1)	TTGGGAGAAGGAGAG CCTGGGAGTCATCGGTAG
NM_008002	Fibroblast growth factor 10 (FGF10)	CCACCATGCTGAAGTGTGTTAG TTTGAGGATTAGGAGGAGGGAAG
NM_008084	Glyceraldehyde 3 phosphate dehydrogenase (GAPDH)	CAGTGAGCTTCCGGTTCA CTGCACCCAGAAG
NM_010495	Inhibitor of DNA binding 1 (Id1)	GCATCTTGTGTCGCTGAG AGGCTGAAAGGTGGAGAG
NM_010496	Inhibitor of DNA binding 2 (Id2)	GCCATTTCACCAGGAGAAC AATAACGGTATCACAGTCCAG
NM_008321	Inhibitor of DNA binding 3 (Id3)	GCATGGATGAGCTTCGATCTTAAC CTTCTCTCGGGCTCCAGGTC

NM_016958	Keratin 14 (Krt1-14)	CCACCTTTCATCTTCCCAATTCTC GTGCGGATCTGGCGGTTG
NM_008470	Keratin 1-16 (Krt1-16)	AATATCCACTCCTCCTCAC GTTGAACCTTGCTCCTTG
NM_010663	Keratin 1-17 (Krt1-17)	ACCTGACTCAGTACAAGCC CCTTAACGGGTGGTCTGG
NM_001042660	MAD homolog 7 (Drosophila) (Smad7)	CGTGTGTCCCGCTTGTCTTG ACTCTCGTTTCCTGTCTTCGTATATG
NM_013601	Msh-like 2 (Msx2)	CGCCTGTTGGACTCTATG TCGCTTAGGGTGACAATG
NM_010849	Myelocytomatosis oncogene (Myc)	TGTACCTCGTCCGATTCC TCTCCTCATGCAGCACTAG
NM_008960	Phosphatase and tensin homolog (Pten)	ATTGTTGGTGTGAGGATGGTAGG AGGTAGGTACGCATTTGCTGAG
AJ459711	Precusor-miR-21	TGTACCACCTTGTCGGATAG GATACCAAAATGTCAGACAGC
AY699265	Primary-miR-21	TCCTAGAATTGGCATTAAGCCCCAG ATGTCACGACCACGACAGGG
NM_001168491	Programmed cell death 4 (Pdcd4)	CCACACTCATACTCTGTTCTTG GCCTCCATCTCCTTCACTTAC
NM_024449	Sclerostin (Sost)	CGGACCTATACAGGACAAG TAGCCCAACATCACACTC
NM_011595	Tissue inhibitor of metalloproteinase 3 (Timp3)	TCGGCTGTTTGGGTTGAG CTCTGGCTGGTGTCTCTTC
NM_024427	Tropomyosin 1 (Tpm1)	AGCATTGATGACTTAGAAGAG GACTTGGAATCTCAGAAGAC

2.7 *In Situ* Hybridization

2.7.1 Principle:

In Situ hybridization (ISH) is a technique, which is applied to localize gene expression at the histological level (Dagerlind *et al.,* 1992). Since the first ISH generally radioactive nucleotides have been used to synthesize probes. One of their major advantages is that radio-labelled probes have the ability to detect very low levels of transcripts. However, their major limitations are relatively poor resolution, safety hazards associated with ionising radiation and long autoradiography exposure times of up to 30 days (Morris *et al.,* 1990). All of these disadvantages are dependent on the radioisotope used in the ISH. Furthermore, the photographic emulsion showing the hybridization signals is not at the same focus as the tissue section, which impairs resolution and microscopic observation (Braissant and Wahli, 1998). The recently use of non-radioactively labelled nucleotides has significantly improved ISH by shortening the amount of time needed for development and provides excellent histological resolution (Morris *et al.,* 1990).

Locked Nucleic Acid (LNA) based detection is currently the most applicable for rare transcripts. These LNA based detection probes have improved significantly the sensitivity to specific miRNA transcripts by increasing the melting temperature of the probes, which provides the bases of stringency required for ISH procedure (Obernosterer *et al.,* 2007). Obernosterer and colleagues (2007) and Silahtaroglu and colleagues (2007), describe optimised methods/conditions for sensitive and specific histological detection of miRNAs by ISH, which were

incorporated into our protocol. These methods were combined with the unique miRNA recognition properties of LNA-modified oligonucleotide probes. The inclusion of LNAs in oligonucleotides dramatically increases the affinity for their RNA targets and enhances mismatch-discrimination capability (Exiqon, Copenhagen, Denmark). LNAs, which are bi-cyclic RNA analogs that allow a significant, increase in the hybridization temperature and thereby an enhanced stringency. Enhanced stringency allowed the use of short probes, which previously could not be used due to reduced stringency and thus limiting their use in ISH (Obernosterer *et al.,* 2007).

2.7.2 *In situ* hybridization procedure:

For miRNA detection on tissue sections, cryosections (10µm) were fixed in 4% paraformaldehyde for 10 min at room temperature. After acetylation in triethanolamine buffer (4.5 mM triethanolamine, 6 M HCl, and 3 mM acetic anhydride) for 10 min and premobilization (1% Triton X-100/1xDEPC treated phosphate buffer-saline) for 30 min, slides were hybridized specifically with DIG-labelled miR-31, miR-21 and miR-214 probes in concentration of 2.5pmol, 12.5pmol and 2.5pmol, respectively, (Exiqon, Copenhagen, Denmark) diluted in hybridization buffer (50% formamide DI, 2xSCC, 1% dextran sulfate, and 0.4 mg/ml t-RNA) for 16-18h at 50°C overnight. Slides subsequently were washed in 2x SCC (10 min, 4 times, 67°C), 0.1x SCC (60 min, 67°C), 0.2x SCC (10 min, RT). Immunodetection of miRNAs was performed with sheep alkaline phosphatase conjugated anti-DIG antibody (1:5000; Roche, Mannheim,

Germany) followed by a staining reaction with NBT/BCIP solution (Roche, Mannheim, Germany) for 16-18h at room temperature.

2.8 Immunohistochemistry

For immunohistochemical analyses, formalin-fixed cryostat sections (10µm) of mouse back skin were used. The cryosections were incubated with primary antisera against Keratin 16 (Abcam, Cambridge, UK; diluted 1:5000), Keratin 17 (Abcam, Cambridge, UK; diluted 1:5000), and Dlx3 (Santa Cruz, CA, USA; diluted 1:100) overnight at +4°C, followed by application of corresponding Alexa-Cy^3 labelled antibody (Invitrogen, UK; diluted 1:200) for 1hr at 37°C. Incubation steps were interspersed by four washes with phosphate buffer-saline (PBS, 5 min each). Image preparation and analysis were performed using a fluorescent microscope (Nikon, Tokyo, Japan) in combination with DS-C1 digital camera and ACT-2U image analysis software (Nikon, Tokyo, Japan).

2.9 Immunocytochemistry

For immunocytochemistry analyses, PMEKs were washed in cold PBS and fixed in methanol (at -20°C for 10mins). These cells were incubated with primary anti-sera against rabbit β-catenin (Abcam, Cambridge, UK; diluted 1:2000), overnight at +4°C, followed by application of anti-rabbit Alexa-Cy^3-labelled antibody (Invitrogen, UK; diluted 1:200) for 1 hour at 37°C. Keratinocytes were counter stained with DAPI (4', 6-diamidino-2-phenylindole). Incubation steps were interspersed by four washes with PBS (5 min each). Image preparation and

analysis were performed using a fluorescent microscope in combination with DS-C1 digital camera and ACT-2U image analysis software.

2.10 Multicolour fluorescence

2.10.1 Fluorescent *in situ* hybridization (FISH) and β-catenin immunofluorescence:

For miR-214 detection on tissue sections (E13-P2.5), cryosections (10µm) were fixed in 4% paraformaldehyde for 10 min at room temperature. After acetylation in triethanolamine buffer (4.5 mM triethanolamine, 6 M HCl, and 3 mM acetic anhydride) for 10 min and premobilization (1% Triton X-100/1xDEPC treated PBS) for 30 min, slides were hybridized with 2.5 pmol DIG-labelled miR-214 probe (Exiqon, Copenhagen, Denmark) diluted in hybridization buffer (50% formamide DI, 2xSCC, 1% dextran sulfate, and 0.4 mg/ml t-RNA) for 16-18h at 50°C overnight. Slides subsequently were washed in 2x SCC (10 min, 4 times, 67°C), 0.1x SCC (60 min, 67°C), 0.2x SCC (10 min, RT). Next, sections were incubated with primary anti-sera against rabbit β-catenin (Abcam, Cambridge, UK; diluted 1:2000) and sheep anti-DIG-conjugated-TRITC antibody (1:50; Roche, Mannheim, Germany) overnight at +4°C, followed by application of anti-rabbit Alexa^{488}-labelled antibody to detect β-catenin (Invitrogen, UK; diluted 1:200) for 1 hour at 37°C. All incubation steps were interspersed by four washes with PBS (5 min each). Image preparation and analysis were performed using a fluorescent microscope in combination with DS-C1 digital camera and ACT-2U image analysis software.

2.11 Flow cytometry analysis

PMEKs were transfected with 200nM anti-miR-31, pro-miR-31, or miRNA negative controls (Dharmacon Inc, USA) as described above (step 2.2.3). After 24 hours of transfection, the medium was removed, and cells were washed twice with PBS, and then trypsinized. Pellets were then fixed in 70% ethanol in PBS (at -20°C for 30 min). The cell suspension was centrifuged at 2000 rpm and resuspended in PBS containing propidium iodide (400µg/ml; Sigma, St. Louis, MO, USA) and RNase A (10 mg/ml; Invitrogen, UK) at 37°C for 30 min. Vials were placed on ice before analysis. Flow cytometry analyses were performed using a FACS-Calibur flow cytometer (BD Biosciences; San Jose, CA, USA). Data obtained were analyzed using the CellQuest software (BD Biosciences; San Jose, CA, USA).

2.12 Alkaline Phosphatase staining

Alkaline phosphatase staining was performed on 10µm cryosections fixed in acetone (at -20°C for 10 mins). The sections were incubated in developing solution (100 mM NaCl pH 8.3, 100 mM Tris, pH 9.5 and 20 mM HCL, 0.05% Naphtol ASBI phosphate, 0.5% DMF, 25 mM Na-Nitrite, and 5% New fuchsin) for 15 mins at room temperature. Sections were then counterstained with haematoxylin for 20s at room temperature, followed by washing with running water.

2.13 Western blot analysis

Proteins were extracted from snap-frozen skin samples or cultured cells using RIPA lysis buffer (**Appendixes B**). Proteins were processed for Western blot analysis by adding 100µl of RIPA lysis buffer to either 1 x 10^7 cells and/or 50mg of tissue. This was followed by homogenisation of mixture by vortexing and spinning down at 1000rpm for 5min at +4°C. The supernatant was removed and Bradford assay was performed to determine protein concentration. Five micrograms of protein were separated on Sodium dodecyl sulphate polyacrylamide (SDS-PAGE) gel electrophoresis at 100 volts for 1hr 30mins. This was followed by transferring proteins (transblotting) to nitrocellulose membrane (Bio-Rad Corp, UK) for 20min at 400mAmps (*for buffers and reagents see* **Appendixes B**). Proteins were incubated with primary antibodies (**Table 2**) overnight at +4°C. Corresponding horseradish peroxidase-tagged IgG antibodies were used as secondary antibody (1:5000; Thermo Scientific, UK). Antibody binding was visualized with an enhanced chemiluminescence's system (SuperSignal West Pico Kit; Thermo Scientific, UK) and autoradiographed with X-ray film (CL-Xposure Film, Thermo Scientific, UK). Densitometric analysis was performed by measuring the intensity of staining of β-actin and target proteins, which in turn correlates with the amount of the protein detected. This was carried out using Total Lab v1.10 software (Biogenetic Services, Brookings, SD, USA).

Table 2: List of primary antibodies used in Western blot

Antigen	Host	Dilution	Manufacturer
Actin	Mouse monoclonal	1:2000	Abcam, Cambridge, UK
FGF10	Goat polyclonal	1:2000	Abcam, Cambridge, UK
K14	Mouse monoclonal	1:3000	Biomeda, Foster City, CA, USA
K16	Rabbit polyclonal	1:3000	Abcam, Cambridge, UK
K17	Rabbit polyclonal	1:3000	Abcam, Cambridge, UK
Scelrostin	Rabbit polyclonal	1:50	Abcam, Cambridge, UK
β-catenin	Rabbit polyclonal	1:2000	Abcam, Cambridge, UK

2.14 Luciferase reporter assay

HaCaT cells were grown in DMEM supplemented with heat-inactivated 10% fetal bovine serum in an atmosphere of 5% CO_2 at 37°C, until 60-70% confluent. 3'UTR fragments containing miRNA specific putative binding sites (**Table 3**) were amplified from mouse genomic DNA using forward and reverse primers containing XhoI and NotI restriction sequences, respectively. The following primers were used:

Table 3: Primer sequences of amplified fragments for reporter gene assays

Target Sequence	Sense/Anti-sense Primers
Keratin 14 3'UTR	GGGCTCGAGAGATCCGCACCAAGGTCAT TTTGCGGCCGCGCAACTCAGAAAAAGAAGC
Keratin 16 3'UTR	GGGCTCGAGGTCCATCCTCAAGGAGCAAG TTTGCGGCCGCCCAAAAAGCTTTATTAGCCTACC
Keratin 17 3'UTR	GGGCTCGAGGCTGCAGAGAGGCAGCTTCCCT TTTGCGGCCGCCGACGTCTCTCCGTCGAAGGGA
FGF10 3'UTR	GGGCTCGAGTGACGATCCAAACATAGAAG TTTGCGGCCGCGCTTTCCAGTAAATGCTTG
Dlx3 3'UTR	AATCTCCCTCCCCTTGCTTA ACACTCTGGCTCCCATTTTG
Sclerostin 3'UTR	GGGCTCGAGTTTCTACACAACAGTTTAAGG TTTGCGGCCGCATTAACAATGCCTCTGGTC.
β-catenin 3'UTR (binding site 1)	CGAGGAGTAACAATACAAATGG TGTTATGTTCTAGTGAACCTG
β-catenin 3'UTR (binding site 2)	GCGGTAGGGTAAATCAGTAAGAG CGCATCTGTTGAAGCATTGTATC
Smad7 3'UTR (binding site 1)	AGTCACAGTATTGCTACC AAGAACAGTGTCAAAGTATC
Smad7 3'UTR (binding site 2)	TGACACTGTTCTTAGCTCAATGAG GTCCTTTCCTCTCTCAAAGCAC

These amplified fragments were cloned at XhoI and NotI sites downstream of CV40 promoter-driven Renilla luciferase cassette in psiCHECK2 (Promega, Madison, WI, USA). For dual luciferase assay, these constructs (200ng) were co-transfected with 200nM pro-miR-31, pro-miR-21, pro-miR-214 or control oligonucleotide (Dharmacon Inc, USA) into HaCaT cells using 0.5 µl Lipofectamine 2000 (Invitrogen, UK) in 96-well plates. As an additional negative control, HaCaT cells were transfected with miRNAs, which did not contain

putative binding sites for gene of interest; such as pro-miR-31 was used with psiCHECK2 plasmids containing Smad7 3'UTR binding site, while pro-miR-21 was used to test the specificity of miR-214 binding to the β-catenin 3'UTR. At 24 hours after transfection, the relative luciferase activities were determined using Dual-Glo® Luciferase Assay System (Promega, Madison, WI, USA) on the Infinite 2000 micro-plate reader (Tecan, Reading, UK).

The effects miR-214 on Wnt/β-catenin mediated transcriptional activation of TCF/Lef binding sites in HaCaT cells was done as described previously (Veeman *et al.,* 2003), using TOPflash plasmid (Addgene, Cambridge, MA, USA; Addgene plasmid 12456). For dual luciferase assay, PMEKS were co-transfected with TOPflash plasmid (200ng) and with 200nM pro-miR-214 or control oligonucleotide (Dharmacon Inc, USA) into HaCaT cells using 0.5 µl Lipofectamine 2000 (Invitrogen, Paisley, UK) in 96-well plates. Four experimental groups were used: 1) untreated cells (control), 2) transfection with control oligonucleotide, 3) treatment with 10mM of LiCl, and 4) combination of transfection with pro-miR-214 and 10mM LiCl treatment. At 24 hours after the beginning of the experiment, the relative luciferase activities were determined using Dual-Glo-luciferase assay system on the Infinite 2000 micro-plate reader.

Chapter III

RESULTS

3.1. Hair cycle-associated changes in the miRNA signature in the skin

3.1.1 MicroRNA microarray profiling

To identify the changes in the miRNA expression in the skin and hair follicle (HF) during distinct stages of the hair cycle in mice (i.e., during anagen or period of active growth and hair production, catagen or stage of apoptosis-driven involution, telogen or period of relative resting), global microarray analysis of miRNAs was performed as described previously (Singh *et al.,* 2008). Total RNA was isolated from the skin of neonatal mice at postnatal days 12-15 (P12-P15), i.e. when HFs complete morphogenesis and actively produce hair (anagen-like stage), as well as at P16-P19 and P20-P23 (i.e., during catagen and telogen stages, respectively) (Paus *et al.,* 1999).

Global miRNA expression profiling revealed the substantial hair cycle-associated changes in the expression of a large number of miRNAs in the skin (**Fig. 6*A***). In particular, expression of 219 out of 568 miRNAs analyzed showed significant ($p<0.01$) differences between the distinct hair cycle stages (**Appendixes A**). Among these miRNAs, a largest proportion showed significant changes in expression between the anagen and telogen skin, while relatively lower number of miRNAs displayed differences in expression between anagen and catagen, as well as between the catagen and telogen stages of the hair cycle (**Fig. 6*B***).

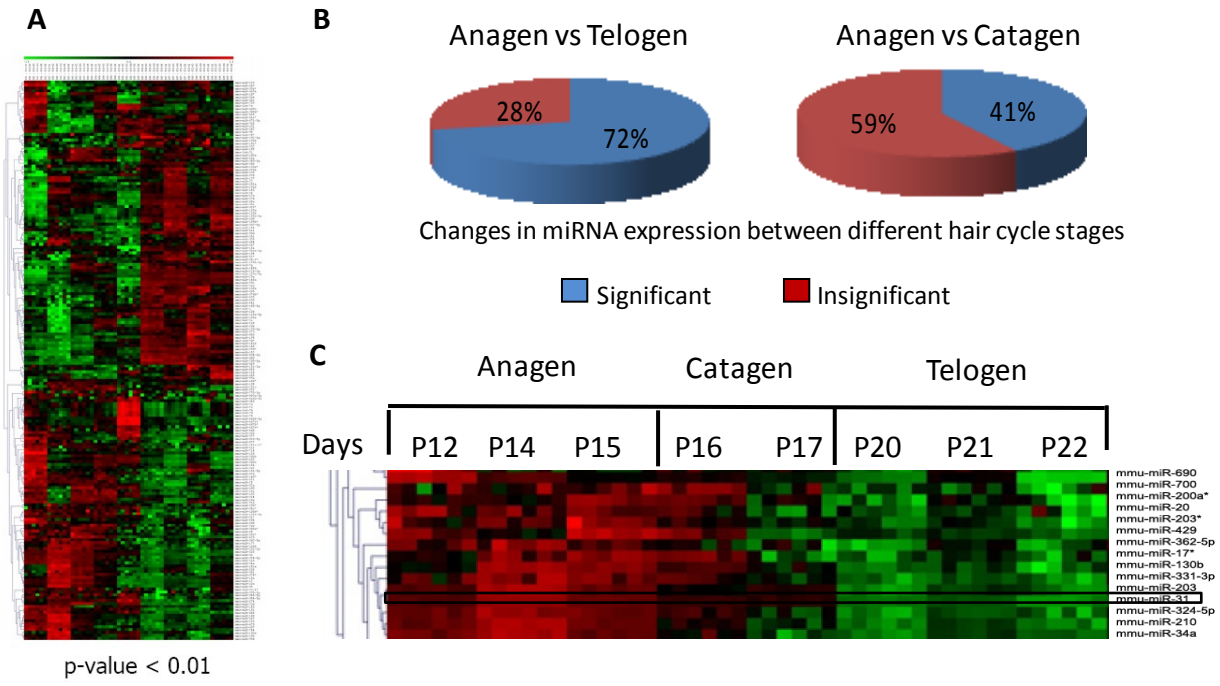


Figure 6. Global miRNA expression profile in mouse skin at distinct hair cycle stages. **A**) Heat map represents differentially expressed miRNAs between distinct stages of the hair cycle. Colour map was used to visualize the difference in expression. **B**) Differences in the expression of miRNAs between distinct hair cycle stages. **C**) Heat map represents a cluster of miRNAs with significantly (P<0.01) up-regulated expression in anagen, compared to catagen and telogen skin. Red colour represents elevated expression of miRNAs. Green represents decreased expression of miRNAs. P <0.01. Unpaired student's *t*- test and ANOVA tests.

3.1.2 A role of microRNA-31 in the skin and hair follicle

For further analyses of the roles of miRNAs in the control of hair cycle-associated gene expression programs in the skin, miR-31 was selected, as its expression showed the most remarkable changes between the anagen and catagen/telogen stages of the hair cycle (**Fig. 6*C***, **Appendixes, A**). Microarray data was validated by qRT-PCR. QRT-PCR showed very high levels of miR-31 transcripts in neonatal skin during the anagen-like stage at P12, while during catagen (P17-P19) its expression progressively decreased ($p<0.02$) and remained at low levels during telogen ($p<0.02$) (P20-P23; **Fig. 7*A***).

Similar changes in the miR-31 levels were observed in adult skin during the depilation-induced hair cycle. MiR-31 expression progressively increased during HF transition from telogen to anagen and reached maximum at late anagen stage (day 12 post depilation) ($p<0.02$), followed by rapid decrease during catagen stage (days 16-19 post depilation) ($p<0.02$) (**Fig. 7*B***).

Consistent with microarray and qRT-PCR data, *in situ* hybridization for miR-31 showed lack of its expression in telogen skin (**Fig.7*C***). MiR-31 expression appeared first in the epidermis and in the growing hair bulb of mid-anagen HF on day 5 post depilation (**Fig. 7*D***). In late anagen HFs (day 12 post depilation), miR-31 was prominently expressed in the hair matrix, as well as in the ORS and IRS, while relatively lower expression was seen in the DP and lack of expression was seen in

the dermis (**Fig. 7*E***). During catagen, miR-31 expression progressively decreased (mid-catagen**; Fig. 7*F***).

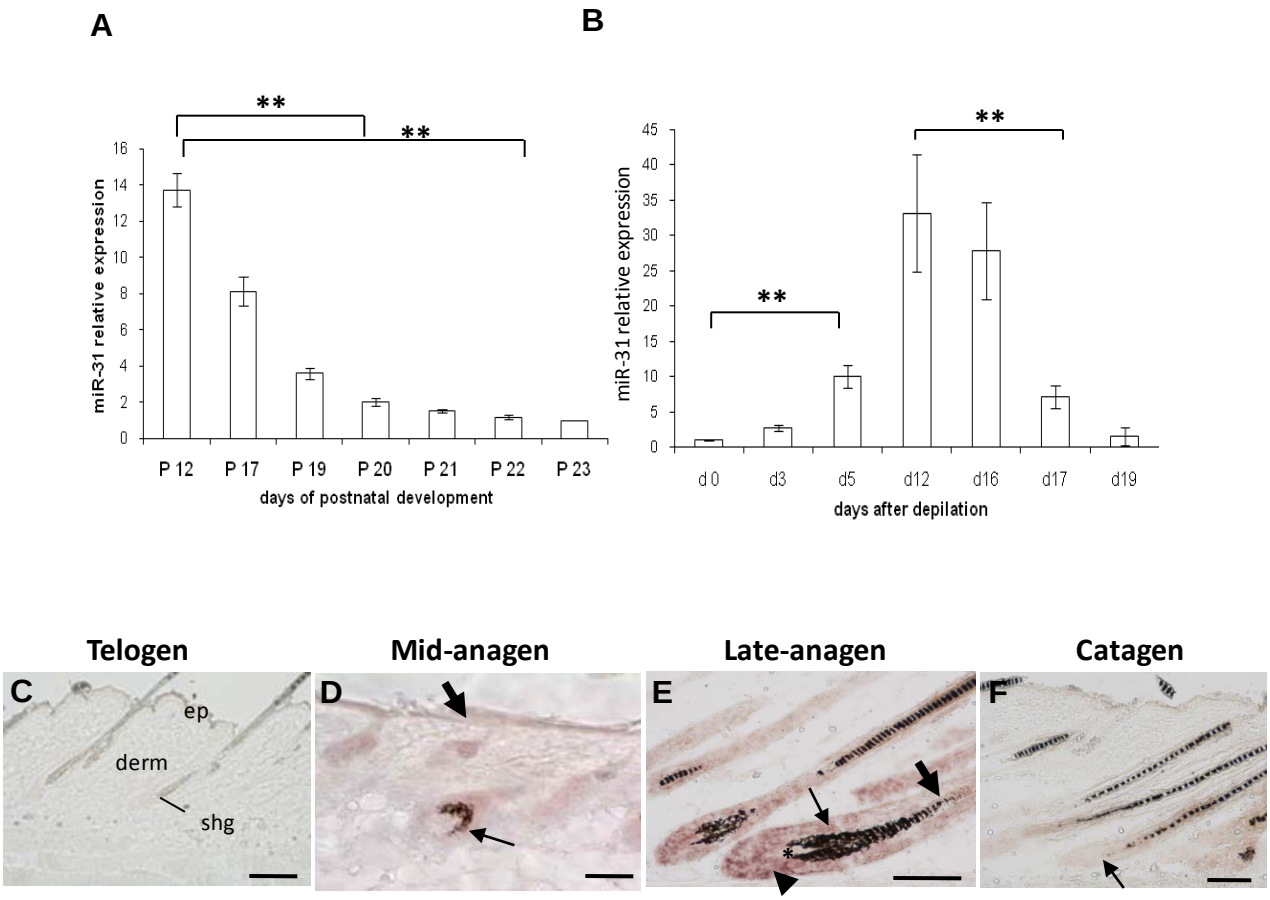


Figure 7. Spatiotemporal expression of miR-31 during hair cycle. A) Detection of miR-31 in neonatal skin by qRT-PCR: it is expressed maximally during the anagen-like stage (P12), while its expression progressively decreased during catagen (P17–P19) and remained at very low levels during telogen (P20–P23). **B**) Detection of miR-31 in the skin during depilation-induced hair cycle: miR-31 expression progressively increased during HF transition from telogen to anagen and reached a maximum at late anagen stage (d12 after depilation), followed by rapid decrease during catagen (d16–19 after depilation). **C-F**) Representative microphotographs of *in situ* hybridization for miR-31 in HF at different hair cycle stages. **C**) Lack of miR-31 expression in telogen. **D**) miR-31 expression in the epidermis (large arrow) and in the growing hair bulb of mid-anagen HF (small arrow). **E**) Prominent miR-31 expression in the hair matrix (arrowhead), outer and inner root sheaths (small and large arrows), and its lower expression in the dermal papilla in late anagen HFs (asterisk). **F**) Low miR-31 expression in the epithelium of catagen HF (arrow). **P <0.02. Unpaired student's *t*- test. *n*= 3. Scale bar 100*μm*. Abbreviations: Ep, epidermis; derm, dermis; shg, secondary hair germ.

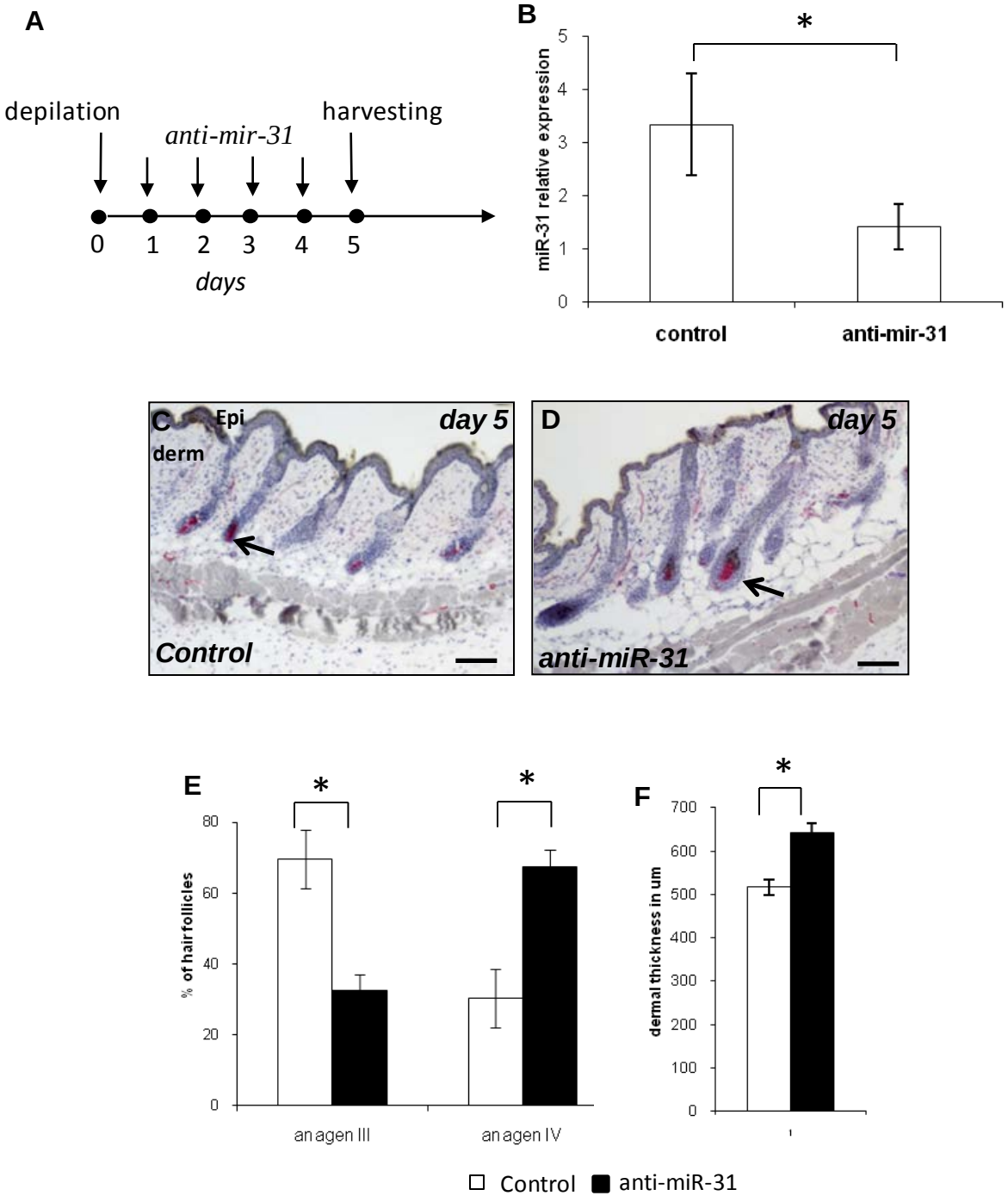
3.1.3 Inhibition of miR-31 activity in the skin accelerates anagen development and alters hair shaft formation

To explore the role of miR-31 in the control of hair cycle, synthetic inhibitor designed to specifically bind to and block the miR-31 activity was administered into the back skin of 7-week-old mice at different time-points of the hair cycle (**Fig. 8*A*, Fig 9*A***). According to the experimental approaches established previously (Zhu *et al.,* 2008), the efficiency of anti-miR-31 in inhibiting the miR-31 activity was assessed by analyzing the expression of miR-31 in the treated and control samples using TaqMan® miRNA assay and qRT-PCR. These experiments showed significant decrease of the miR-31 expression in the skin treated with miR-31 inhibitor ($p<0.05$) versus the control (**Fig. 8*B***)

In the first experiment, anti-miR-31 was administered daily into mouse back skin during the early anagen (days 1-4 of the depilation-induced hair cycle), and skin was harvested at day 5 after the beginning of the experiment (**Fig. 8*A***). Inhibition of miR-31 activity during early anagen resulted in acceleration of anagen progression compared to the control (**Fig. 8*C,D***). In mice treated with anti-miR-31, significantly more HFs were found in anagen IV stage ($p<0.05$), characterizing by larger and more pigmented hair bulbs, whereas the majority of HFs in the control skin reached only anagen III phase of the hair cycle (**Fig. 8*C-E***). Acceleration of anagen development in mice treated with anti-miR-31 versus the controls was also associated with significant ($p<0.05$), morphologically recognizable increase in the

skin thickness (**Fig. 8*F***), used as an additional well-established parameter of the hair cycle progression in mice (Handjiski *et al.,* 1994).

In the second experiment, mice were treated with anti-miR-31 during the mid-anagen phase of the hair cycle (days 4-7 post depilation), and skin samples were collected on day 8 of the experiment (**Fig. 9*A***). The efficiency of miR-31 inhibition in skin was assessed by analyzing the expression of miR-31 using TaqMan® miRNA assay and qRT-PCR in the treated and control samples. These experiments showed significant decrease ($p<0.05$) of the miR-31 expression levels in the skin treated with miR-31 inhibitors against the control (**Fig. 9*B***). Administration of anti-miR-31 during mid-anagen did not cause any significant changes in the rate of hair cycle progression, and HFs in both experimental and control skin reached anagen VI stage. However, HFs treated with anti-miR-31 were characterized by larger hair bulbs, altered hair shaft structure with irregular distribution of melanin, and hyperplastic and deformed ORS, compared to the control (**Fig. 9*C,D***).


A
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anti-mir-31
harvesting
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days
B
*
miR-31 relative expression
0 1 2 3 4 5
control
anti-mir-31
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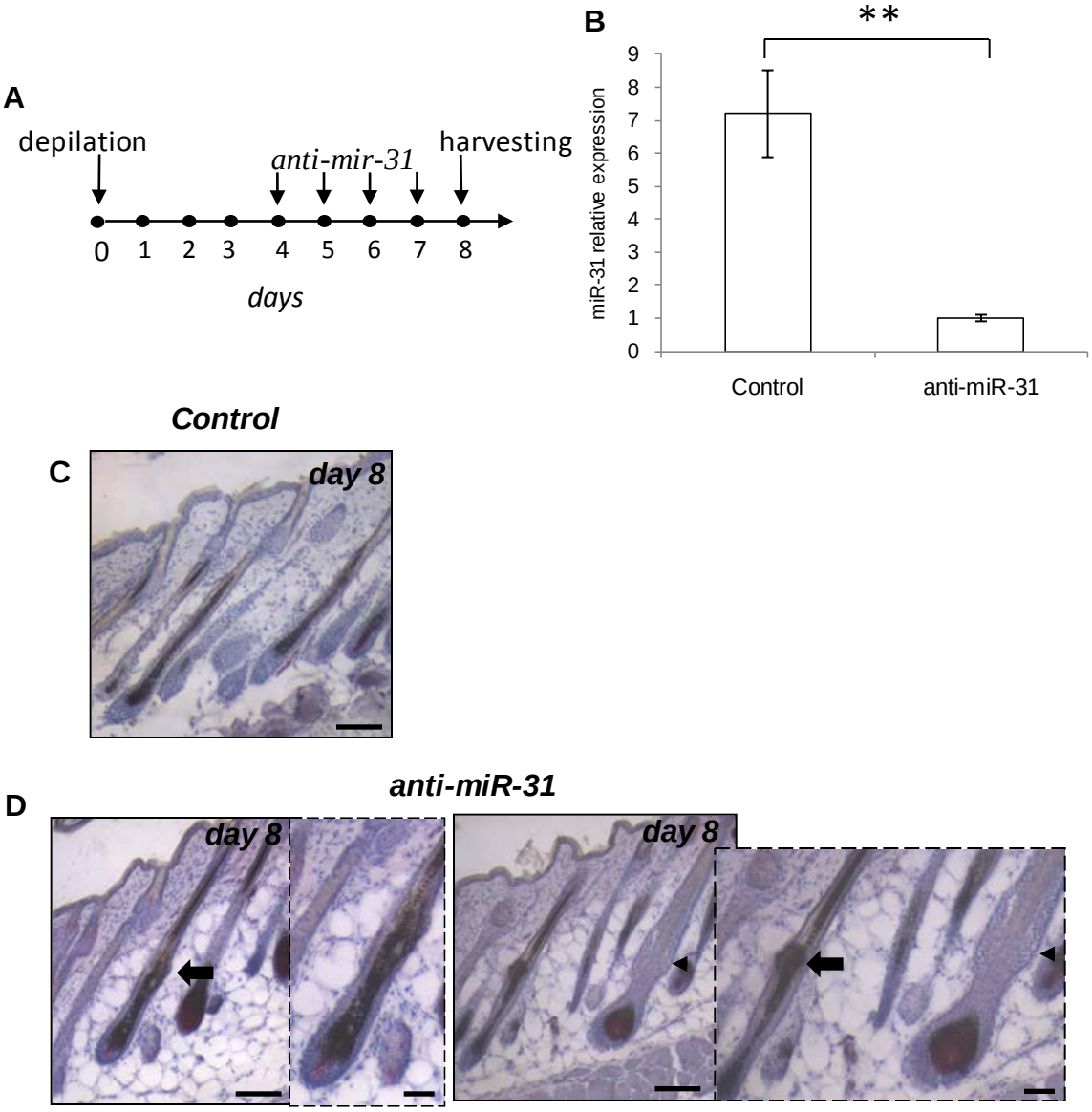
Epi
derm
day 5
Control
D
day 5
anti-miR-31
E
*
*
% of hairfollicles
0 20 40 60 80
anagen III
anagen IV
F
*
dermal thickness in um
0 100 200 300 400 500 600 700
Control
anti-miR-31

Figure 8. Inhibition of miR-31 accelerates early anagen development. A-E) Hair cycle was induced by depilation in the back skin of 8-week-old C57BL/6 mice; anti-miR-31 or vehicle control were administered daily subcutaneously at dpd 1-4. Skin was harvested at dpd 5 (**A**). **B**) Inhibition of miR-31 activity was assessed by TaqMan real-time PCR: a significant reduction in miR-31 levels was detected in treated skin compared with control. **C-D**) Representative skin examples of control (**C**) and anti-miR-31-treated mice at day 5 (**D**); sections were stained for the detection of endogenous alkaline phosphatase activity to visualize the morphology of dermal papilla as an important indicator of the defined stages in HF cycle. Percentage of HFs in defined stages of anagen was evaluated in cryostat sections of the skin of control or anti-miR-31-treated mice by quantitative histomorphometry using established morphological criteria (Muller-Rover *et al.,* 2001); there was a significant increase in the percentage of HFs in anagen IV stage in anti-miR-31-treated skin, compared with the control (**E**). **F**) Skin thickness after anti-miR-31 treatment is significantly enhanced, compared to the control. *$P < 0.05$. Unpaired student's *t*- test. $n = 3$. Scale bar 100μm. Abbreviations: Epi, epidermis; derm, dermis.


A
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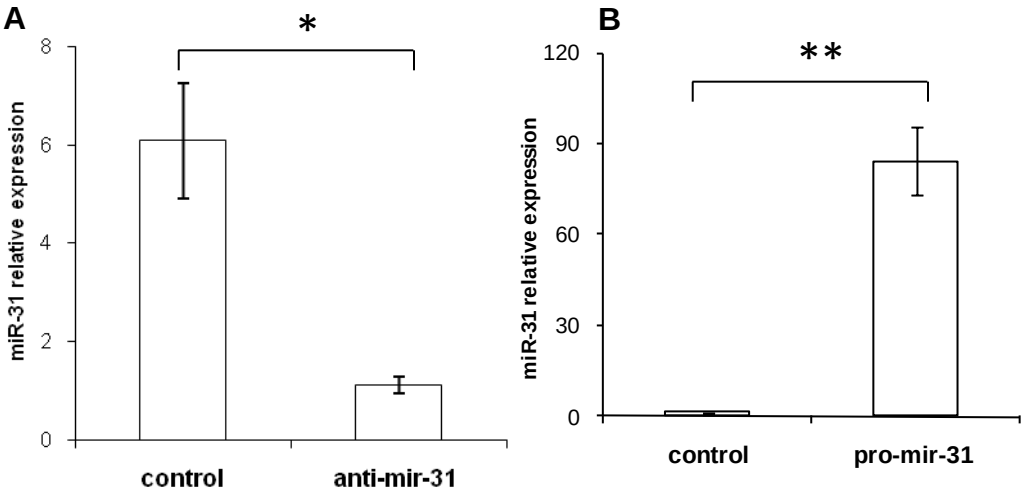
anti-mir-31
harvesting
0 1 2 3 4 5 6 7 8
days
B
**
miR-31 relative expression
9 8 7 6 5 4 3 2 1 0
Control
anti-miR-31
Control
C
day 8
anti-miR-31
D
day 8
day 8

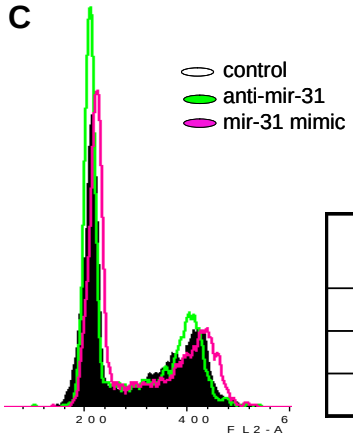
Figure 9. Inhibition of miR-31 during mid-anagen phase alters hair follicle morphology. Hair cycle was induced by depilation in the back skin of 8-week-old C57BL/6 mice; anti-miR-31 or vehicle control were administered daily subcutaneously at dpd 4–7. Skin was harvested at dpd 8 (**A**). **B**) Inhibition of miR-31 activity was assessed by TaqMan real-time PCR: a significant reduction miR-31 levels was detected in treated skin compared with control. Representative skin examples of control and anti-miR-31-treated mice (**C,D**) at day 8. **C**) Representative skin example of control skin showing HFs with anagen VI morphology. **D**) Microphotographs of HFs that received anti-miR-31 treatment, depicting hair shaft deformation (large arrows), hyperplastic and deformed outer root sheath (arrowheads) of the HF after anti-miR-31 treatment. ***P* <0.02. Unpaired student's *t*- test. *n*= 3. Scale bar 100*μm*.

3.1.4 Does modulation of the miR-31 activity lead to changes in cell cycle progression in keratinocytes?

To further explore mechanisms underlying the effects of miR-31 on hair cycle, primary mouse epidermal keratinocytes (PMEKs) were transfected either with anti-miR-31 or with miR-31 mimic (pro-miR-31) to inhibit or enhance miR-31 activity, respectively. Transfection efficiency was assessed by qRT-PCR, which revealed significant decreased ($p<0.05$) and increased ($p<0.02$) in the miR-31 levels in the keratinocytes after treatment with anti-miR-31 or pro-miR-31, respectively, versus the controls (**Figure 10*A,B***).

To assess whether miR-31 shows any effects on cell proliferation, PMEKs were stained by propidium iodide and flow cytometry analysis was performed. FACS quantification did not reveal any significant changes in cell cycle progression as a result of either miR-31 inhibition or its activation (**Fig. 10*C***). This data suggest miR-31 activity is not involved in the regulation of keratinocyte proliferation.





	control	anti-mir-31	mir-31 mimic
G1	54.3	54.1	53.8
S	11.6	10.3	12.7
G2/M	33.6	34.9	31

Figure 10. FACS analysis of miR-31 effects on cell cycle progression. (**A,B**) qRT-PCR analysis revealed significant increase and decrease in expression levels of miR-31 in keratinocytes treated with anti-miR-31 or pro-miR-31, respectively, compared with the controls. **C**) No changes in cell cycle progression through the different phase of cell cycles as a result of either miR-31 inhibition or activation was detected by FACS analysis. *P < 0.05, **P < 0.02. Unpaired student's t- test. n= 3.

3.1.5 Modulation of miR-31 activity induces complex changes in gene expression program in primary mouse epidermal keratinocytes

To identify putative target genes for miR-31, PMEKs transfected with anti-miR-31, were processed for microarray analysis, using Whole Mouse Genome 60-mer oligo-microarray (manufactured by Agilent Technologies). Global gene expression analysis in keratinocytes treated with anti-miR-31 or control cells revealed that miR-31 inhibition resulted in two-fold or higher changes in expression of 419 genes that encode distinct adhesion molecules, components of the cytoskeleton, metabolic enzymes and growth factors/receptors/signalling molecules involved in the control of cell fate decision and differentiation (**Fig. 11,** for complete list of genes see **Appendixes C**). Functional assignments of the genes, which expression was altered in keratinocytes after anti-miR-31 treatment revealed that 53% of them represent distinct signalling and growth regulatory molecules or transcription factors, thus suggesting a potential involvement of miR-31 in the control of keratinocyte responsiveness to growth factor stimulation/inhibition during anagen development and hair cycle-associated tissue remodelling.

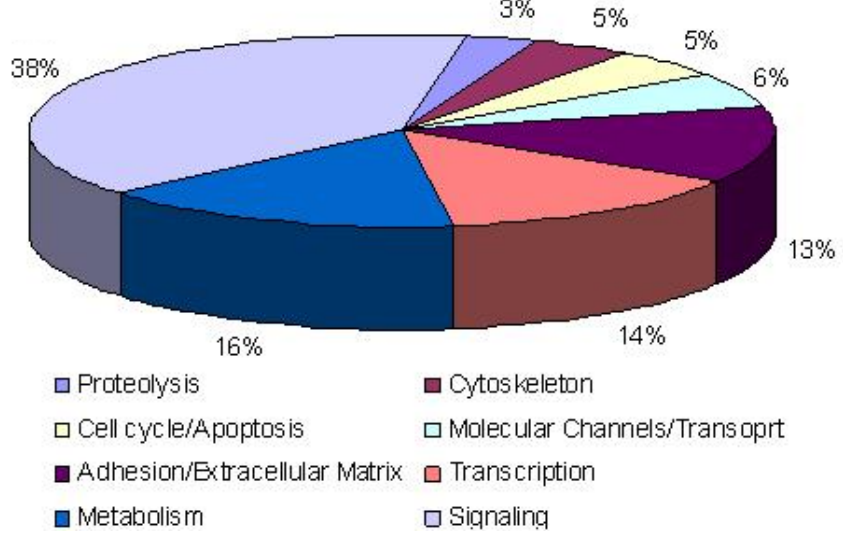


Figure 11. Functional annotation of the genes differently expressed in primary mouse epidermal keratinocytes treated with sequence specific antagonist of miR-31. Analysis of the microarray data demonstrated two-fold or higher changes in expression of >400 genes encoding for adhesion/extracellular matrix molecules, cell differentiation, metabolism, signalling, transcription, cell cycle/apoptosis and cytoskeleton markers.

Gene	Symbol	Increased in fold expression
Transcription factors		
Distal-less homeobox 3	Dlx3	2.4
Distal-less homeobox 1	DLx1	2.1
T-box 15	Tbx15	2
Peroxisome proliferative activated receptor alpha	PPARa	2.7
Peroxisome proliferative activated receptor gamma	PPARg	2.5
Signalling		
Sclerostin	Sost	3.3
Fibroblast growh factor 14	Fgf14	2.5
Mitogen-activated protein kinase 15	Mapk15	2.5
Mitogen-activated protein kinase kinase kinase 2	Map3k2	2.4
Mitogen-activated membrane bound inhibitor	Bambi	2.2
Fibroblast growh factor 10	Fgf10	2.0
Adhesion		
FAT tumour suppressor homolog 3	Fat3	2.2
Neural cell adhesion molecule 1	Ncam	2.0
Cadherin 17	Cdh17	2.0
Structural Proteins		
Keratin 14	Krt 14	3.0
Keratin 16	krt 16	2.4
Keratin 17	krt 17	3.4
Keratin 5	krt 5	2.5
Keratin 6a	krt 6a	2.6

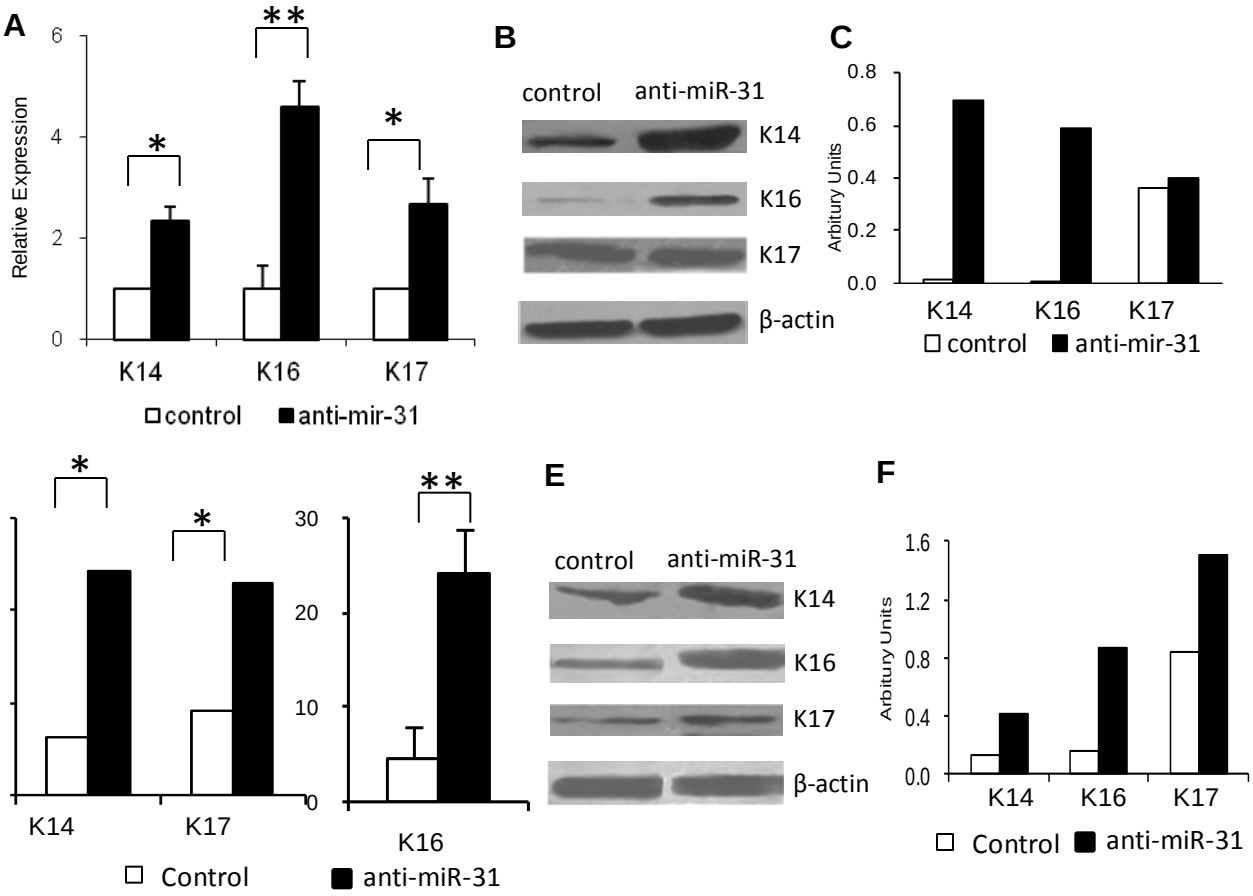
Table 4. Selection of over expressed genes in mice skin treated with sequence specific antagonist of microRNA-31. Analysis of the microarray data was used to select genes, which showed two-fold or higher changes in expression after miR-31 inhibition in keratinocytes. These genes are known key regulators in hair follicle development, maintenance and regeneration.

3.1.6 MicroRNA-31 controls the expression of keratin 14, 16 and 17 in the hair follicle

In addition to the changes in expression of genes that encode signalling/regulatory molecules and transcription factors implicated in hair cycle control, microarray analysis revealed that expression of several keratin genes has been affected after inhibition of the miR-31 activity (**Table 4**). Transcripts for keratins 14, 16 and 17 (*Krt14*, *Krt16*, and *Krt17*, respectively) were increased in the keratinocytes treated with anti-miR-31, compared to the controls (**Fig. 12*A***). Treatment of the keratinocytes with anti-miR-31 also resulted in increase in the levels of keratins 14, 16, but not keratin 17 proteins (K14, K16, K17, respectively) (**Fig. 12*B***). Moreover, administration of anti-miR-31 into mouse back skin also resulted in upregulation of Krt14, Krt16, and Krt17 expression. Elevated levels of *Krt14*, *Krt16*, and *Krt17* transcripts and corresponding proteins were detected in the extracts of the full-thickness skin treated with anti-miR-31 versus the controls, as determined by qRT-PCR and Western blotting, respectively (**Fig. 12*D,E***).

K16 and K17 expression by immunofluorescence showed marked alterations in the HFs treated with anti-miR-31 versus the control (**Fig. 12*G-J*).** Consistent with data reported previously (Panteleyev *et al.,* 1997), K17 immuno-reactivity was detected in the unilateral cluster of hair matrix cells, as well as in the ORS and in the medulla of the hair shaft of control HFs (**Fig. 12*G***). However, anti-miR-31 administration resulted in the increase of K17 expression in the unilateral cluster of matrix cells

and in the opposite side of the hair matrix, as well as in the hair shaft and thickened ORS versus the controls (**Fig. 12*H***). K16 expression also increased in the ORS and companion layer of the HF after anti-miR-31 treatment compared to the controls (**Fig. 12*I,J***).


A
Relative Expression
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*
*
K14
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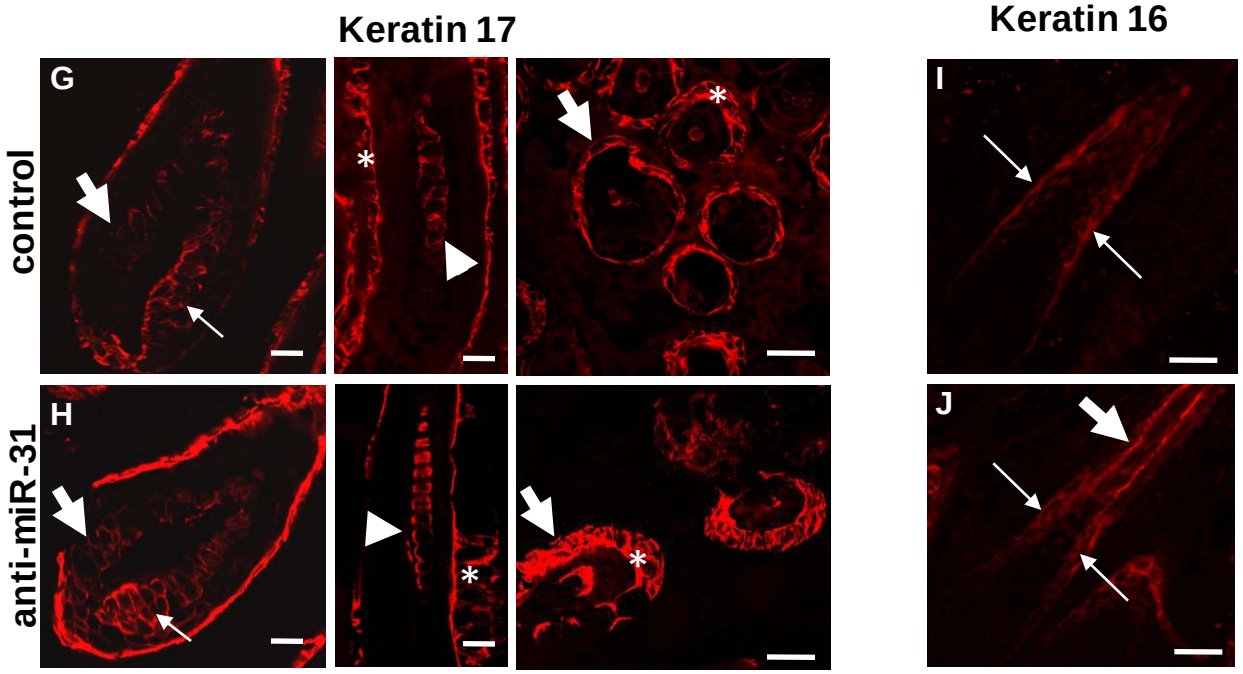
anti-miR-31


Keratin 17
Keratin 16
control
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G
H
I
J

Figure 12. MiR-31 regulates expression of keratins in the primary mouse epidermal keratinocytes and in mouse skin. Keratin (K)14, K16, and K17 expression in the primary keratinocytes transfected with either anti-miR-31 or control oligonucleotides: qRT-PCR analysis showing a significant upregulation of K14, K16, and K17 mRNA levels in the keratinocytes transfected with anti-miR-31 (**A**); Western blot analysis demonstrating increased levels of K14, K16, but not K17 proteins after transfection with anti-miR-31(**B**) confirmed by the densitometric analysis (**C**). Expression of K14, K16, and K17 in skin treated either with anti-miR-31 or with vehicle control: a significant up-regulation of K14, K16, and K17 mRNA in skin that received anti-miR-31 treatment compared with control was detected by qRT-PCR (**D**). Western blot analysis shows elevated K14, K16, and K17 protein levels after anti-miR-31 treatment (**E**), confirmed by the densitometric analysis (***F***). **G,H**) K17 detection by immunofluorescence in the control and anti-miR-31 treated skin, respectively. Increase in K17 expression in the unilateral disk (small arrow) and in the opposite side of the hair matrix (large arrow) in the treatment compared with the control. Prominent K17 expression was also seen in the hair shaft (arrowhead) and in the thickened outer root sheath (asterisk) in anti-miR-31 treated skin (**H**) compared to the hair shaft (arrowhead) and outer root sheath (asterisk) in the control (**G**). **I, J**) K16 immunofluorescence in the HF: increased immunoreactivity of K16 in the outer root sheath and companion layer of the HF after anti-miR-31 treatment (**J**), (small arrows and large arrow, respectively), compared to the controls (small arrows) (**I**). **P* >0.05; ***P* >0.02. Unpaired student's *t*- test. *n*= 3. Scale bar 100*μm*.

3.1.7 MicroRNA-31 regulates expression of the distinct components of the FGF, BMP signalling pathways and the Dlx3 transcription factor in the skin

Expression of the selected genes whose expression in keratinocytes was changed after anti-miR-31 treatment (**Table 4**) and which are implicated in the control of hair cycle and keratinocyte differentiation (fibroblast growth factor 10 (Fgf10), bone morphogenetic protein, and activin membrane bound inhibitor (Bambi), Sclerostin (Sost), and distal-less homeobox 3 (Dlx3) (Stenn and Paus, 2001; Botchkarev, *et al.,* 2003; Alonso and Fuchs, 2006) was further examined in mouse back skin treated with anti-miR-31. Consistent with microarray data, the anti-miR-31 treatment resulted in increased expression of transcripts for Fgf10, BMP pathway inhibitor Bambi, Wnt, and BMP signalling antagonist Sost (**Fig. 13*A***). Western blot analysis of the full-thickness skin samples obtained after anti-miR-31 treatment *in vivo* also showed that Fgf10 and Sost protein levels were increased compared to the controls (**Fig. 13*B***). Expression of the Dlx3 transcription factor was also increased in primary keratinocytes treated with anti-miR-31 (**Fig. 13*D***). By immunofluorescence analysis of the skin treated with anti-miR-31, ectopic Dlx3 expression in the HF ORS and increase of the Dlx3 expression in the hair matrix, IRS, and hair shaft were seen (**Fig. 13*F***) compared with the controls (**Fig. 13*E***). These data suggest that the effects of the anti-miR- 31 on anagen progression and hair shaft formation (**Fig. 8, Fig. 9**) were executed, at least in part, via modulation of the activity of the Fgf, BMP, and Wnt pathways and Dlx3 transcription factor in the HFs.

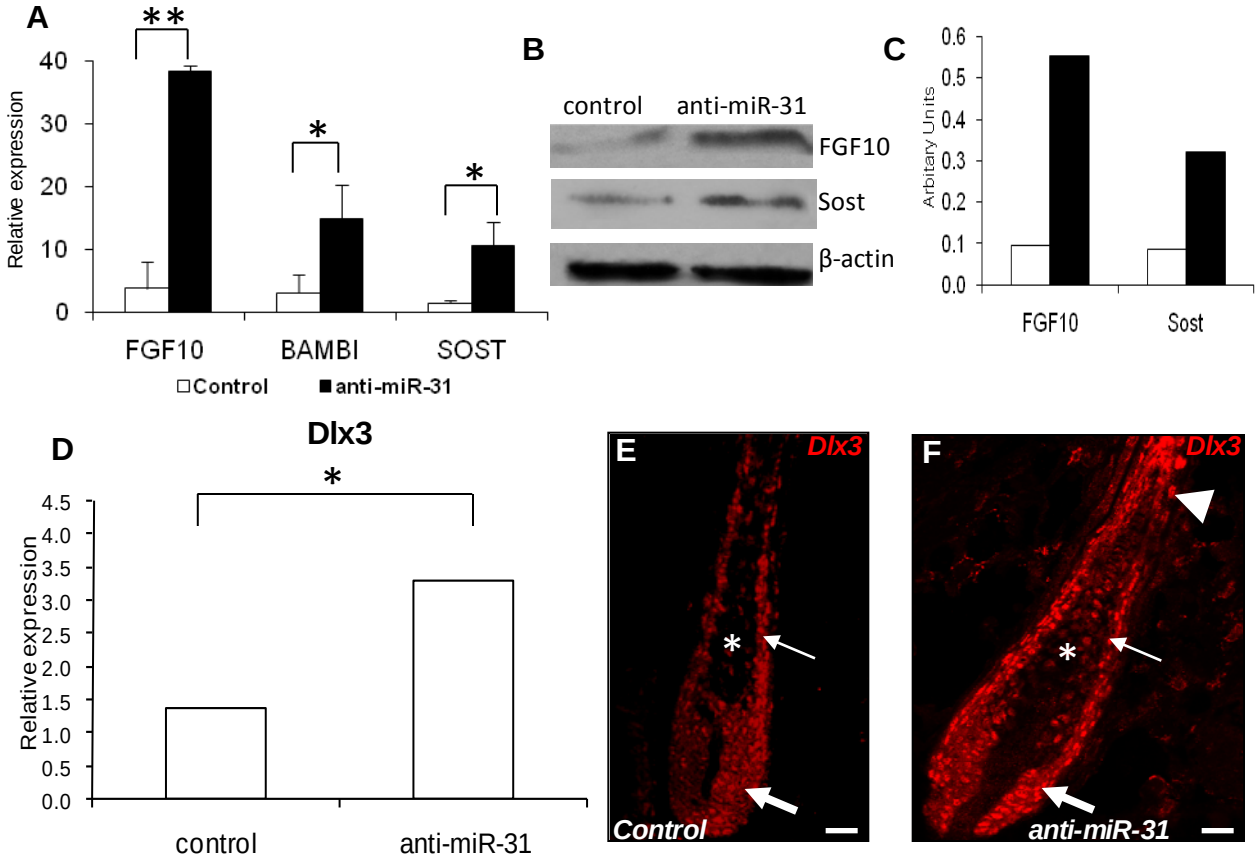


Figure 13. Changes in gene expression of FGF10, BAMBI, Sost and Dlx3 in the skin due to miR-31 inhibition. A) Elevated expression of FGF10, BAMBI, and SOST transcripts in anti-miR-31-treated skin compared with the controls was detected in the extracts of the full-thickness skin by qRT-PCR. **B**) Western blotting assay of the extracts of the full-thickness skin either treated with anti-miR-31 or with vehicle control to detect FGF10 and Sost proteins: up-regulation of FGF10 and Sost proteins was detected in anti-miR-31-treated samples compared with the controls confirmed by densitometric analysis (**C**). **D**) Detection of Dlx3 transcripts by qRT-PCR showed its up-regulation in anti-miR-31-treated skin compared with the control. **E,F**) Analysis of Dlx3 protein expression by immunofluorescence. In the control, Dlx3 is expressed in the hair matrix (large arrow), inner root sheath (small arrow), and in the hair shaft (asterisk) (**E**); In anti-miR-31 skin, elevated expression was observed in the hair matrix (large arrow), inner root sheath (small arrow), and in the hair shaft (asterisk), Dlx3 is present in the outer root sheath (arrowhead) (**F**). **P* <0.05; ***P* <0.02. Unpaired student's *t*- test. *n*= 3. Scale bar 50*μm*.

3.1.8 Keratin 16, Keratin 17, FGF10 and Dlx3 are direct targets of miR-31

Bioinformatic analysis using RNA22 algorithm revealed that Dlx3, Fgf10, Sost, Krt14, Krt16, and Krt17 carry several putative miR-31 binding sites (**Fig 14*A***). To validate whether miR-31 directly regulates expression of these genes, the effects of miR-31 on the 3'UTR (un-translated region) target mRNA were tested using a luciferase reporter assay. We judged the repression of average luciferase activity by 30% or more as a significant effect of miRNA on gene expression (Miranda *et al.,* 2006).

Co-transfection of HaCaT cells with pro-miR-31 and the Krt16 3'UTR reporter construct caused 50% reduction in luciferase activity, compared to the corresponding control (**Fig. 14*B***). A putative binding site within the 3' UTR of K17 introduced into reporter construct co-transfected with pro-miR-31, decreased luciferase activity by 60%, which contained a single-mismatched hexamer found within the 3'UTR (**Fig. 14*B***). Greater than 60% suppression of the luciferase activity was also observed after co-transfection of the cells with pro-miR-31 and Dlx3 3'UTR reporter construct, compared to the control (**Fig. 14*B***). A putative binding site within the 3'UTR of *Fgf10* mRNA introduced into the luciferase reporter construct also demonstrated sensitivity to miR-31 in the co-transfection assay by reducing luciferase activity greater than 30% (**Fig. 14*B***).

However, the reporter constructs containing segments of the 3'UTRs of Krt14 and Sost did not demonstrate significant sensitivity to miR-31 in the co-transfection

experiments (**Fig. 14*B***). Collectively, these data indicate that Krt16, Krt17, Dlx3, and Fgf10, but not Krt14 and Sost transcripts might represent genuine targets of miR-31.

Summary

MiR-31 is predominantly expressed in the HF during its active growth phase. Inhibition of miR-31 leads to: the accelerated development of early anagen, hyperplastic changes in the ORS and hair shaft defect during mid-anagen phase. MiR-31 regulates HF differentiation and cycling by i) involvement in the cross-talk between the BMP and Wnt signalling pathways ii) modulating FGF10 pathway iii) regulation of expression of structural genes Krt16 and Krt17. By targeting a number of growth regulatory molecules, transcription factors and cytoskeletal proteins, miR-31 is involved in the control of hair cycling and hair fiber formation.

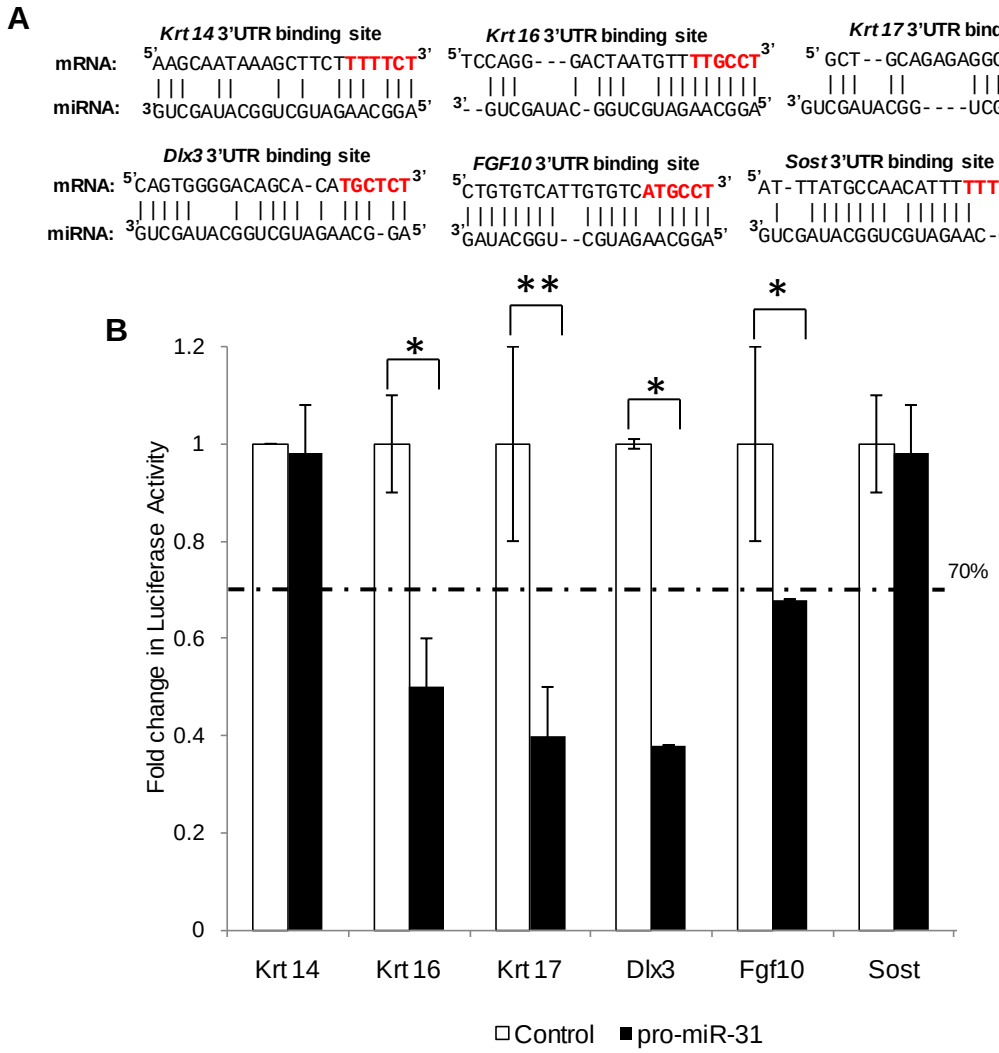


Figure 14. Krt16, Krt17, Dlx3 and FGF10 are primary targets of miR-31. A) Predicted interactions between miR-31 and *Krt14*, *Krt16, Krt17, Dlx3, Sost,* and *Fgf10* mRNA. Alignment of mouse sequences in the 3'-UTR of *Krt14*, *Krt16, Krt17, Dlx3, Sost* and *Fgf10* mRNA. The seed region is shown in red. Representation is limited to the region around the miR-31 complementary site. **B**) Cotransfection of HaCaT cells with pro-miR-31 and the Krt16 3'UTR, Krt17 3'UTR, Dlx3 3'UTR construct encompassing putative binding sites caused more than 40% reduction in luciferase activity, compared to the corresponding controls. About 30% suppression in the luciferase activity has also been observed after co-transfection of the cells with pro-miR-31 and Fgf10 3'UTR reporter construct, compared to the control. Pro-miR-31 did not cause significant reporter inhibition with constructs containing segments of the 3'UTR of *Krt14* and *Sost* mRNA. *$P < 0.05$, **$P < 0.02$. Unpaired student's *t*- test. $n = 3$.

3.2 Exploring the role of microRNA-214 in skin biology

Based on miRNA microarray profiling, we also selected miR-214 for further investigation, because its expression levels showed significant changes between the growth phase (anagen) and catagen/telogen transitional stages of the hair cycle.

To validate microarray data qRT-PCR was performed and showed very high levels of miR-214 transcripts in neonatal skin during the anagen-like stage at P12, while during catagen (P17-P19) its expression significantly decreased ($p<0.02$), and during telogen stage expression remained at low levels ($p<0.02$) (P20-P23; **Fig. 15**).

To determine miR-214 localisation during adolescent HF cycle, *in situ* hybridization was employed. In telogen skin, miR-214 expression was observed in the epidermis, HF infundibulum, SHG and the bulge (**Fig. 16*A***). During mid-anagen (day 5 post depilation), miR-214 expression remained in the epidermis and HF bulge, as well as appeared in the HF bulb, ORS and DP (**Fig. 16*B***). In late anagen HFs (day 12 post depilation), miR-214 was prominently expressed in the hair matrix, IRS and ORS, while relatively lower expression was detected in the DP (**Fig. 16*C***). During catagen (day 18 post depilation), prominent expression of miR-214 was still seen in the regressing hair matrix, while its expression decreased in the epithelial sheaths and in the epidermis (**Fig. 16*D***).

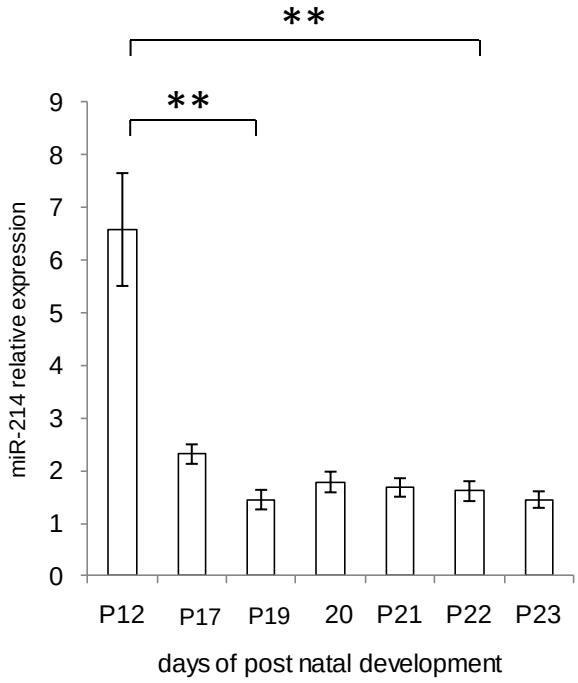


Figure 15. QRT-PCR analysis of miR-214 expression levels in mouse neonatal skin. MiR-214 is expressed maximally during the anagen-like stage (P12), while during catagen (P17-P19) its expression progressively decreased and remained at low levels during telogen (P20-P23). ***P* < 0.02. Unpaired student's *t*- test. *n*= 3.

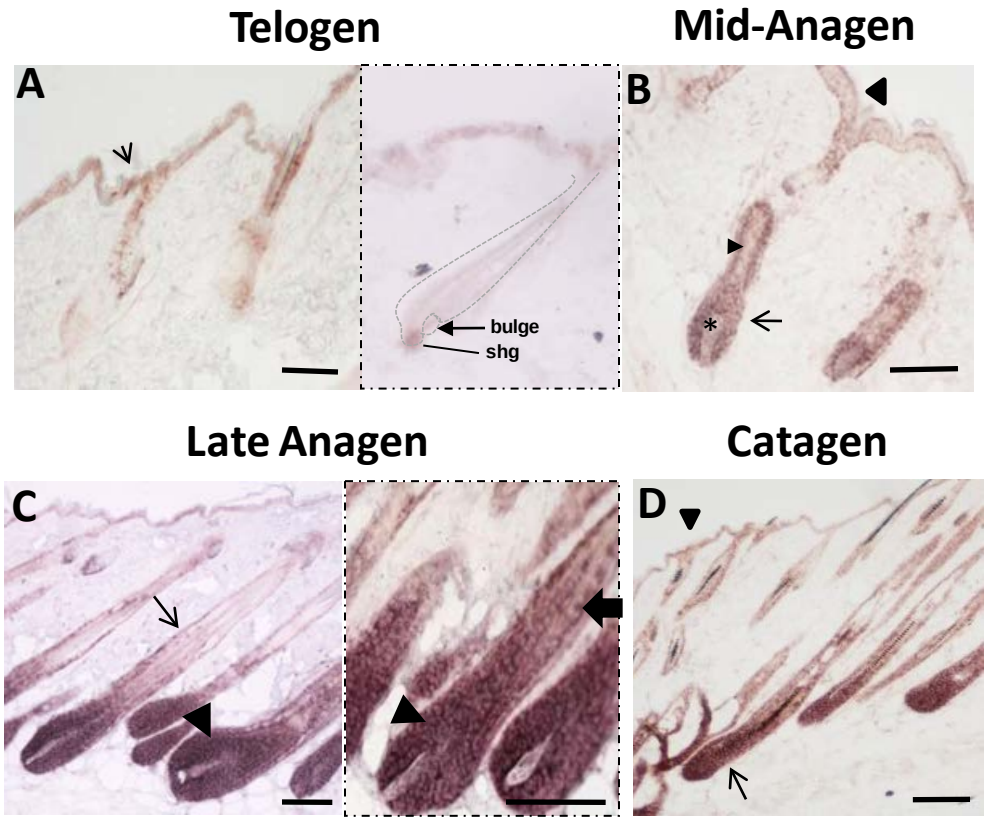


Figure 16. Spatio-temporal expression of microRNA-214 during the hair cycle. A-D) Representative microphotographs of *in situ* hybridization for miR-214 in the HF at different hair cycle stages. **A**) Expression of miR-214 was detected in the epidermis (arrowhead), the bulge and secondary hair germ. **B**) Expression of miR-214 was detected in the epidermis (large arrowhead), hair matrix (arrow) and no expression was detected in the pre-cortex of the HF (small arrowhead) and dermal papilla (asterisk) in mid-anagen HFs. **C**) Prominent expression of miR-214 was seen in the hair matrix (arrow heads), with lower expression was observed in the outer and inner root sheaths (small and large arrows, respectively) and dermal papilla, in late-anagen HFs. **D**) During catagen, weaker expression of miR-214 was seen in the epidermis (arrowhead) and epithelial sheaths, while expression remained elevated in the hair matrix (arrow), which included the dermal papilla. Scale bar 100*μm*.

3.2.1 Involvement of miR-214 in the regulation of the activity of canonical Wnt signalling pathway.

Bioinformatic analysis using RNA22 algorithm, a method for identifying miRNA binding sites and their corresponding heteroduplexes (Miranda *et al.,* 2006) revealed that β-catenin carries two putative miR-214 binding sites in its 3' UTR region. Moreover, all the miR-214 seed regions (2-8nt position) and corresponding pairing sites make perfect and/or near perfect complementarity with 3'UTR of target mRNA (**Fig. 18*D,E***), which is a common accepted principle in miRNA target prediction (Brennecke *et al.,* 2005).

β-catenin is an important component of the Wnt signalling pathway. In the course of Wnt signalling, cytoplasmic β-catenin becomes translocated into the nucleus, where it binds to Lef/TCF transcription factors, which leads to activation of Wnt target gene transcription (Miller *et al.,* 1999). β-catenin is a multifunctional protein that plays an important role during skin and HF morphogenesis and cycling (Huelsken *et al.,* 2001; Miller, 2002) and regulates stem cell differentiation (Huelsken *et al.,* 2001). Therefore, delineating the relationship and regulatory mechanisms between miR-214 and β-catenin will shed some light on the role of miR-214 in the control of normal skin and HF development and HF growth and cycling.

3.2.2 Expression of microRNA-214 and β-catenin during hair follicle morphogenesis

Fluorescent *in situ* hybridization was used to analyze the expression patterns and possible co-localisation of miR-214 and β-catenin during HF morphogenesis. Examination of expression of β-catenin and miR-214 in early embryonic skins (E13) mice revealed β-catenin presence throughout the epithelium surface, whereas miR-214 expression was not detected during this stage (**Fig. 17A,*B)***. At E17.5, the epidermis and hair placodes were β-catenin positive (stage 1, large arrow and asterisk **Fig. 17*E*,*F***). In contrast, low expression of miR-214 was detected in the epidermis and hair placodes (stage 1, small arrows). In the HFs at stages 3-4 of HF development, miR-214 expression was detected, while β-catenin expression was absent from these HFs (arrowheads, **Fig.17*D*,*F***). During the next phase of follicle development (stage 5), β-catenin expression was detected in the central stalk of the developing HF, whilst its expression was more prominent in the epidermis (**Fig. 17*H*,*I***). In contrast, miR-214 expression was seen in the peripheral regions of these developing HF (**Fig. 17*G*,*I***). In more advanced stages of HF development (stage 6), β-catenin expression increased in the stalk of the developing HFs (**Fig. 17*K*,*L***), which coincided with a dramatic reduction miR-214 expression in these HFs (**Fig. 17*J***).

This data suggests that miR-214 may govern the expression patterns and levels of β-catenin during HF induction and development.

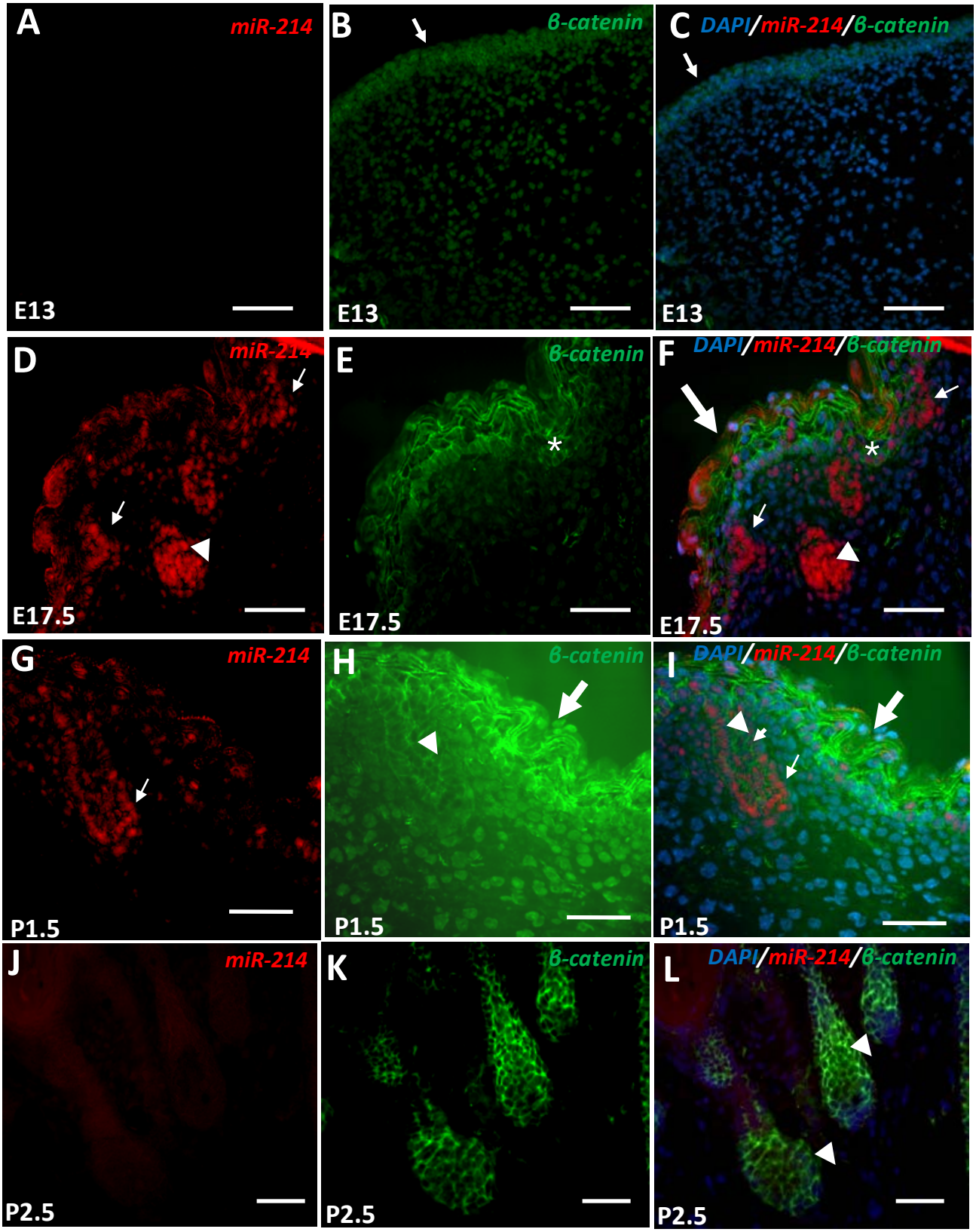

A
miR-214
E13
B
β-catenin
E13
C
DAPI/miR-214/β-catenin
E13
D
miR-214
E17.5
E
β-catenin
E17.5
F
DAPI/miR-214/β-catenin
E17.5
G
miR-214
P1.5
H
β-catenin
P1.5
I
DAPI/miR-214/β-catenin
P1.5
J
miR-214
P2.5
K
β-catenin
P2.5
L
DAPI/miR-214/β-catenin
P2.5

Figure 17. Expression of miR-214 and β-catenin during HF morphogenesis. A-L) Representative microphotographs of fluorescent *in situ* hybridization for miR-214 and β-catenin immunofluorescence during hair follicle development. **A-C**) miR-214 was not detected in E13 skin in the epidermis (**A,C**). β-catenin expression was detected in the uniform basal keratinocyte layer of the epidermis (arrows) (**B,C**). Low expression of miR-214 was detected in early stages of HF development (stage 1, small arrows), while higher expression of miR-214 was detected in HFs in more advanced stages (stages 3-4, arrowheads) (**D,F**). β-catenin expression was detected in the epidermis (large arrows) and in hair placodes (stage 1, asterisks) (**E-F**). With further progression of HF morphogenesis, β-catenin was expressed in differentiating keratinocytes of the developing HFs (stage 5, arrowheads), while expression remained elevated in the epidermis (large arrows) (**H,I**). MiR-214 expression reduced in the epidermis and increased in the peripheral regions of the developing HFs (small arrows), with relative low expression detected in the central parts of the HF (**G,I**). β-catenin expression increased in the central differentiating keratinocytes of the developing HFs (stage 6, arrowheads), with relatively lower expression detected in the epidermis (**K-L**). No expression of miR-214 was detected in these developing HFs (stage 6) (**J,L**). Scale bar 100μm.

3.2.3 MicroRNA-214 regulates β-catenin expression in primary mouse epidermal keratinocytes

To define if miR-214 is involved in the regulation of β-catenin expression, PMEKs were transfected with miR-214 mimic (pro-miR-214) to enhance miR-214 activity. Transfection efficiency was assessed by qRT-PCR, which revealed significantly increased ($p<0.01$) levels of miR-214 transcripts in the PMEKs treated with pro-miR-214 versus the control (**Fig. 18*A***).

QRT-PCR and Western blot analysis were employed to detect the changes in β-catenin expression in the keratinocytes in response to elevated miR-214 activity. This resulted in the level of *β-catenin* transcript being significantly reduced ($p<0.05$) after pro-miR-214 transfection compared with the control (**Fig. 18*B***). Western blot analysis of the PMEKs samples obtained after pro-miR-214 transfection showed β-catenin protein levels were also decreased compared to the control keratinocytes (**Fig. 18*C***).

To validate whether miR-214 directly regulates expression of β-catenin, the effects of miR-214 on β-catenin 3'UTR was tested using a luciferase reporter assay. We judged the repression of average luciferase activity by 30% or more as a significant effect of miRNA on gene expression (Miranda *et al.,* 2006). Two predicted putative binding sites within the 3'UTR of *β-catenin* mRNA were separately introduced into a luciferase reporter gene and caused different effects on luciferase activity (**Fig. 18*D,E***). The first putative binding site within the 3'UTR of *β-catenin* mRNA

introduced into the luciferase reporter construct showed no effect on luciferase activity (**Fig. 18*D***). In contrast, co-transfection of HaCaT cells with the β-catenin 3'UTR construct encompassing the second putative binding site, showed more than 60% reduction in luciferase activity (**Fig. 18*E***). To assess whether this reduction of luminescence was specific, transfection of β-catenin 3'UTR construct (binding site 2) was carried out with alternative miRNA, which does not have any putative binding sites for β-catenin, such as miR-21. Co-transfection of HaCaT cells with miR-21 mimic and the β-catenin 3'UTR construct had no effect on luciferase activity (**Fig. 18*E*).** These data suggest that β-catenin carry one functional binding site for miR-214, therefore it serves as a direct target of miR-214.

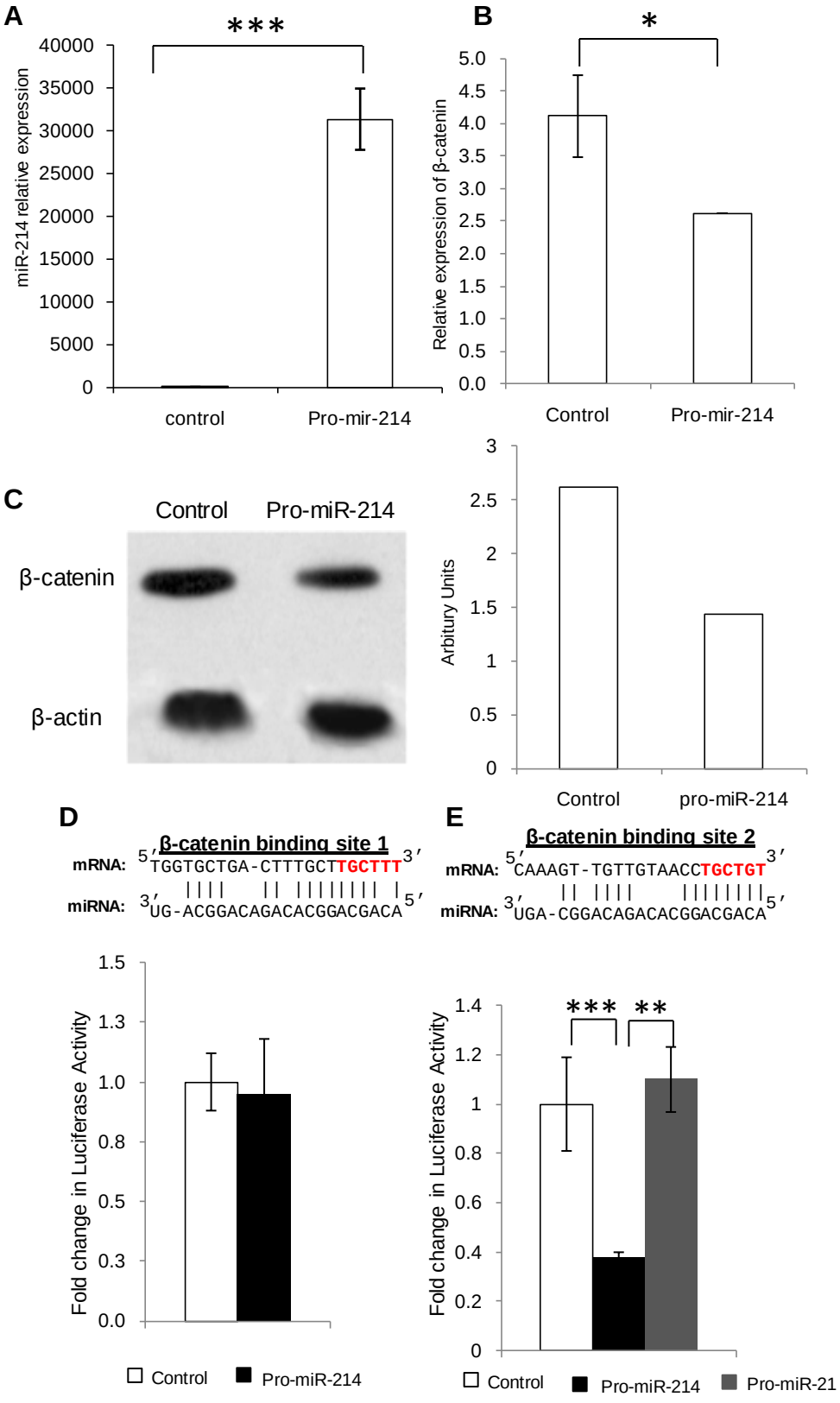

A
***
miR-214 relative expression
40000
35000
30000
25000
20000
15000
10000
5000
0
control
Pro-mir-214
B
*
Relative expression of β-catenin
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Control
Pro-mir-214
C
Control
Pro-miR-214
β-catenin
β-actin
Arbitury Units
3
2.5
2
1.5
1
0.5
0
Control
pro-miR-214
D
β-catenin binding site 1
mRNA: 5′ TGGTGCTGA-CTTTGCTTGCTTT 3′
miRNA: 3′ UG-ACGGACAGACACGGACGACA 5′
E
β-catenin binding site 2
mRNA: 5′ CAAAGT-TGTTGTAACCTGCTGT 3′
miRNA: 3′ UGA-CGGACAGACACGGACGACA 5′
Fold change in Luciferase Activity
1.5
1.3
1.0
0.8
0.5
0.3
0.0
Control
Pro-miR-214
Fold change in Luciferase Activity
***
**
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Control
Pro-miR-214
Pro-miR-21

Figure 18. MiR-214 regulates expression of β-catenin in keratinocytes. A) MicroRNA-214 levels was assessed by qRT-PCR: significant increase in the miR-214 levels in the keratinocytes after treatment with pro-miR-214 compared with the controls. **B**) qRT PCR: β-catenin transcripts were significantly reduced in keratinocytes treated with pro-miR-214 compared with control. **C**) Western blotting assay of the extracts of keratinocytes treated with pro-miR-214 or with vehicle control: a decrease in β-catenin protein was detected in pro-miR-214-treated cells compared with the control confirmed by densitometric analysis. **D,E**) Predicted interaction between miR-214 and *β-catenin* mRNA. The seed region is shown in red. The first putative binding site within the 3'UTR of *β-catenin* mRNA introduced into the luciferase reporter construct showed no effect on luciferase activity (**D**), while co-transfection of HaCaT cells with the β-catenin 3'UTR construct encompassing the second putative binding site, showed more than 60% reduction in luciferase activity (**E**), compared to controls and miR-21. *P* *<0.05, **P <0.02, ***P < 0.01. Unpaired student's *t*- test. n= 3.

3.2.4 Effect of miR-214 on the Wnt/β-catenin signalling pathway

To further explore if miR-214 expression is involved in regulating Wnt-mediated effects, PMEKs were treated with lithium chloride for the activation of Wnt signalling. Lithium chloride acts as a potent inhibitor of GSK-3 protein. GSK-3 protein is part of the 'destruction complex' required for the degradation of cytoplasmic β-catenin (Klein and Melton, 1995). The consequence of the activation of the canonical Wnt/β-catenin pathway is the activation and free, signalling pool of stable β-catenin protein in the cell that enters the nucleus and forms a complex with members of the Lef/TCF family of transcription factors, resulting in increased expression of target genes (Miller *et al.,* 1999; Persad *et al.,* 2001; Stambolic, 2002). This can only be achieved by regulating the stability of β-catenin in the cell (Miller *et al.,* 1999).

Immunocytochemistry was used to analyze the effects of miR-214 on β-catenin intracellular localisation in the keratinocytes. By immunocytochemistry, β-catenin was localised predominantly in the cytoplasm of the untreated keratinocytes (**Fig. 19*C***). In contrast, lithium chloride treatment resulted in translocation of β-catenin into the nucleus of keratinocytes (**Fig. 19*D***). However, transfection of keratinocytes with pro-miR-214 prevented lithium chloride-induced translocation of β-catenin into the nucleus (**Fig. 19*E***).

As the next approach, we analyzed expression of *Axin2* as an additional indicator of Wnt signalling activity (Jho *et al.,* 2002, Lui *et al.,* 2000). In our experiments we

observed that the transcript levels of Axin2 were significantly higher ($p<0.05$) in lithium chloride treated cells compared to the control (untreated) cells. However, transfection of keratinocytes with pro-miR-214 abrogated the lithium chloride-induced upregulation of *Axin2* transcripts ($p<0.02$) (**Fig. 19*A***).

Next, the effects of miR-214 on Lef/TCF-activity was analysed by using luciferase reporter plasmid, TOPflash. Transfection of HaCaT cells with TOPflash and their subsequent stimulation with lithium chloride resulted in significant increase in the luciferase activity, compared to the controls, including untreated cells ($p<0.05$) and cells transfected with control oligonucleotides ($p<0.02$). However, elevated TOPflash reporter activity after lithium chloride treatment was abrogated by co-transfecteion with pro-miR-214 ($p<0.01$) (**Fig. 19*F***).

In addition, we analyzed the effect of miR-214 on genes that are downstream targets of Wnt/β-catenin signalling. We selected *Cyclin D1*, *c-myc* and *Pten* genes. Interestingly, Pten has already been proven to be target of miR-214 (Yang *et al.,* 2008). Treatment of the keratinocytes with pro-miR-214, resulted in the marked decrease in mRNA levels for *c-myc*, *cyclin D1* and *Pten* ($p<0.01$, $p<0.01$, $p<0.05$, respectively) versus controls (**Fig. 19*B***).

These results indicate that miR-214 may indeed negatively regulate Wnt signalling pathway activity in the keratinocytes by targeting β-catenin.

Summary

MiR-214 is predominantly expressed in the anagen HFs during the HF cycle. MiR-214 is a negative regulator of Wnt signalling. MiR-214 and β-catenin are mutually exclusively expressed during HF morphogenesis and β-catenin was confirmed as a direct target of miR-214. Therefore, miR-214 may regulate Wnt mediated effects in HF morphogenesis, HF growth and cycling.

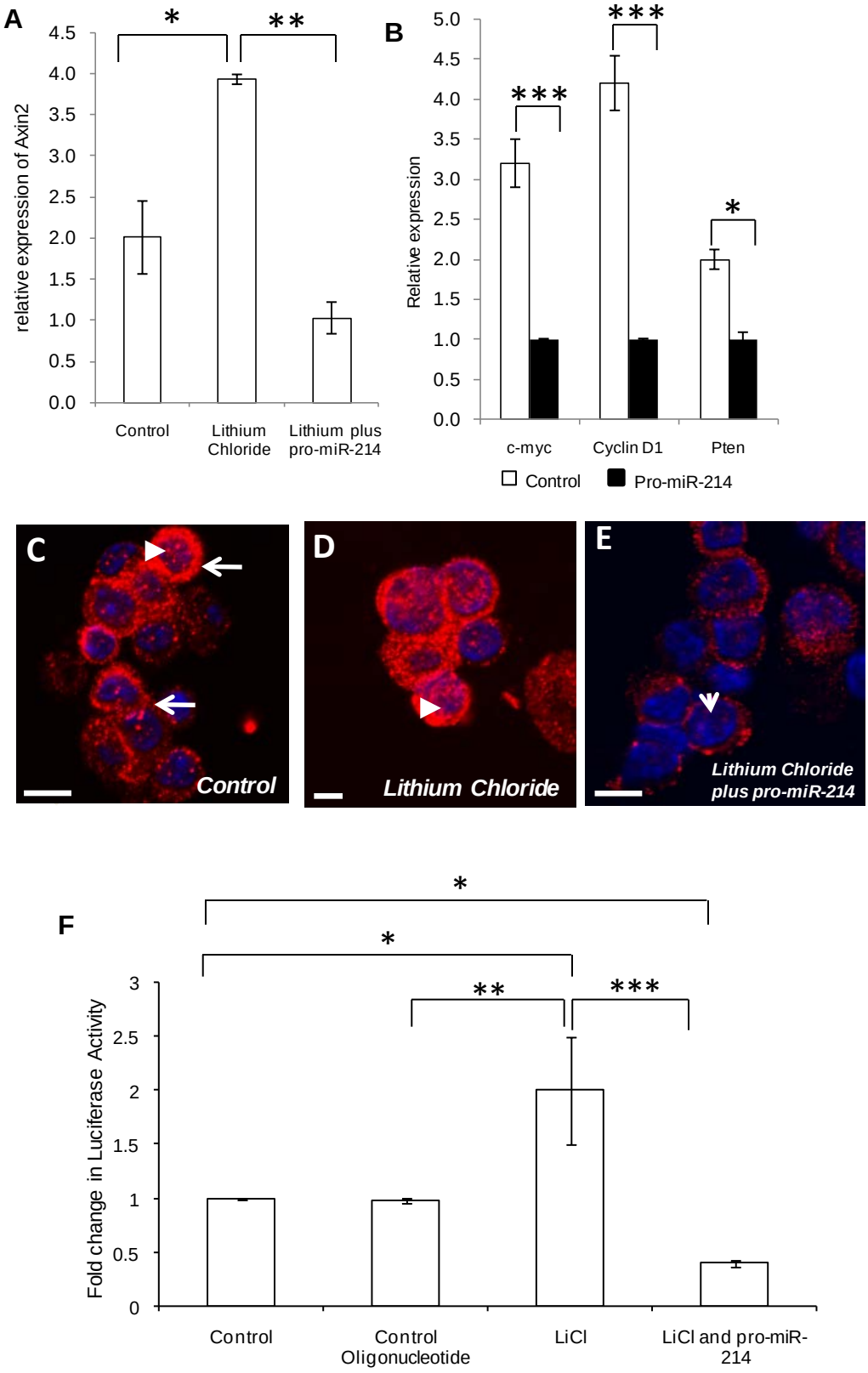

A
relative expression of Axin2
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
**
Control
Lithium Chloride
Lithium plus pro-miR-214
B
Relative expression
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
***
***
*
c-myc
Cyclin D1
Pten
Control
Pro-miR-214
C
Control
D
Lithium Chloride
E
Lithium Chloride plus pro-miR-214
F
Fold change in Luciferase Activity
3
2.5
2
1.5
1
0.5
0
*
*
**
***
Control
Control Oligonucleotide
LiCl
LiCl and pro-miR-214

Figure 19. MiR-214 regulates Wnt signalling activity in the primary mouse epidermal keratinocytes. A) Activation of Wnt signaling in keratinocytes was carried out using lithium chloride (LiCl) at 10mM concentration. qRT PCR revealed significant increase in the Axin2 levels in keratinocytes treated with LiCl. However, transfection of keratinocytes with pro-miR-214 abrogated the LiCl-induced upregulation of Axin2 transcripts. **B**) miR-214 decreases expression of c-myc, cyclin D1 and Pten transcripts in keratinocytes compared with controls. **C-E**) Immunocytochemistry was used to analyze the effects of miR-214 on β-catenin intracellular localisation in the keratinocytes. In untreated cells, β-catenin was primarily localsied in the cytoplasm of cells (arrows), with some cells showing translocation of β-catenin into cell nucleus (arrowhead) (**C**). Keratinocytes treated with LiCl showed localization of β-catenin in the nucleus (arrowhead) (**D**). Keratinocytes treated with LiCl plus pro-miR-214 showed a dramatic reduction in β-catenin localization in the nucleus of cells (arrowhead) (**E**). **F**) The effects of miR-214 on Lef/TCF-activity was analysed by using luciferase reporter plasmid, TOPflash. Transfection of HaCaT cells with TOPflash and their subsequent stimulation with LiCl resulted in significant increase in the luciferase activity, while TOPflash reporter activity after LiCl treatment was abrogated by co-transfection in keratinocytes with pro-miR-214. *P < 0.05, P **< 0.02, ***P < 0.01. Unpaired student's t- test. n= 3. Scale bar 100μm.

3.3 BMP4-induced changes in the expression of miRNAs in keratinocytes

It is widely accepted that miRNAs regulate their targets via interaction and binding to the 3'UTR of target genes. However, little is known about the mechanism regulating miRNAs expression.

BMP signalling plays essential roles during skin development, postnatal tissue remodelling, and tumourigenesis (Li *et al.,* 2006). BMP signalling inhibits the initiation of the HF development, as well as operates as tumour suppressor in adult skin (Botchkarev *et al.,* 1999; Sharov *et al.,* 2009). The goal of this study was to explore if some of the inhibitory effects of BMP signalling are mediated by miRNAs.

To identify the changes in the miRNA expression in response to the activation by BMP4, global microarray analysis of miRNAs was performed as described previously (Singh *et al.,* 2008). Total RNA was isolated from the PMEKs treated with BMP4 for 4 hours. Global miRNA expression profiling revealed that 42 out of 226 miRNAs analyzed showed significant ($p<0.01$) changes in their expression in response to BMP4 treatment (**Fig. 20**). However, only 13 from those 42 miRNAs showed changes in ≥2 fold or higher (**Table 5**). We selected miR-21, whose expression was significantly decreased after exposure to BMP4, compared to the control for further analysis.

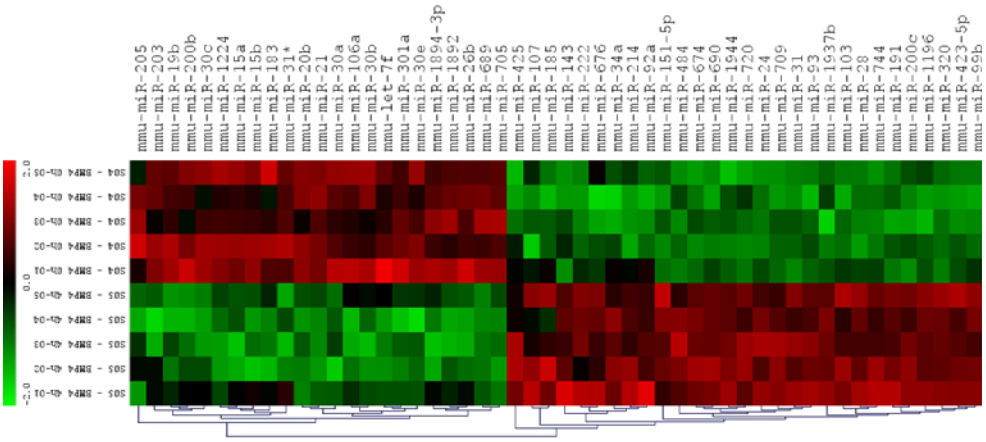


Figure 20. Global miRNA expression profile in primary mouse epidermal keratinocytes after BMP-4 treatment. Heat map represents substantial changes in expression of miRNAs in control keratinocytes compared with keratinocytes treated with BMP4. Colour map was used to visualize the difference in miRNA expression. Red colour represents elevated expression of miRNAs. Green represents decreased expression of miRNAs. P <0.01. Unpaired student's *t*- test and ANOVA tests.

MicroRNA	BMP4 0h	BMP4 4h	Fold change	p-value
miR-1196	500	1,252	2.5	3.00E-04
miR-1944	340	706	2.1	7.89E-05
miR-744	230	471	2.0	2.17E-04
miR-674	323	631	2.0	4.48E-06
miR-28	222	411	1.9	2.10E-05
miR-30a	520	294	-1.8	4.15E-04
miR-1894-3p	706	334	-2.1	2.62E-03
miR-21	**23,698**	**9,392**	**-2.5**	**4.40E-06**
miR-705	1,684	648	-2.6	1.86E-05
miR-689	2,378	829	-2.9	1.46E-04
miR-1892	473	163	-2.9	5.38E-04
miR-26b	303	90	-3.4	8.55E-04
miR-20b	1,079	253	-4.3	2.06E-04

Table 5. Changes in microRNA gene expression program in primary keratinocytes due to BMP4 treatment. Microarray analysis of the global microRNA expression profiling in keratinocytes; ≥2-fold or higher changes in expression of miRNAs after BMP4 treatment.

3.3.1 BMP4 inhibits expression of miR-21 in primary mouse epidermal keratinocytes

To validate microarray data and explore further if BMP4 inhibits miR-21 expression and interferes with its transcription, we analysed expression of primary precursor-miR-21 (pri-miR-21), stem-loop precursor-miR-21(pre-miR-21), and mature form of miR-21 by real-time PCR analysis.

Levels of pri-miR-21 and pre-miR-21 in the keratinocytes remained unchanged after either 4 or 12 hours of BMP4 treatment (**Fig. 21*A***). However, dramatic decrease in the expression of mature miR-21 form was detected in response to BMP4 exposure, compared to the control (**Fig. 21*B***). This data suggest that BMP4 negatively regulates expression of miR-21 post-transcriptionally, involving only in the final step of its biogenesis, cytoplasmic processing of pre-miR-21 into mature miR-21 form.

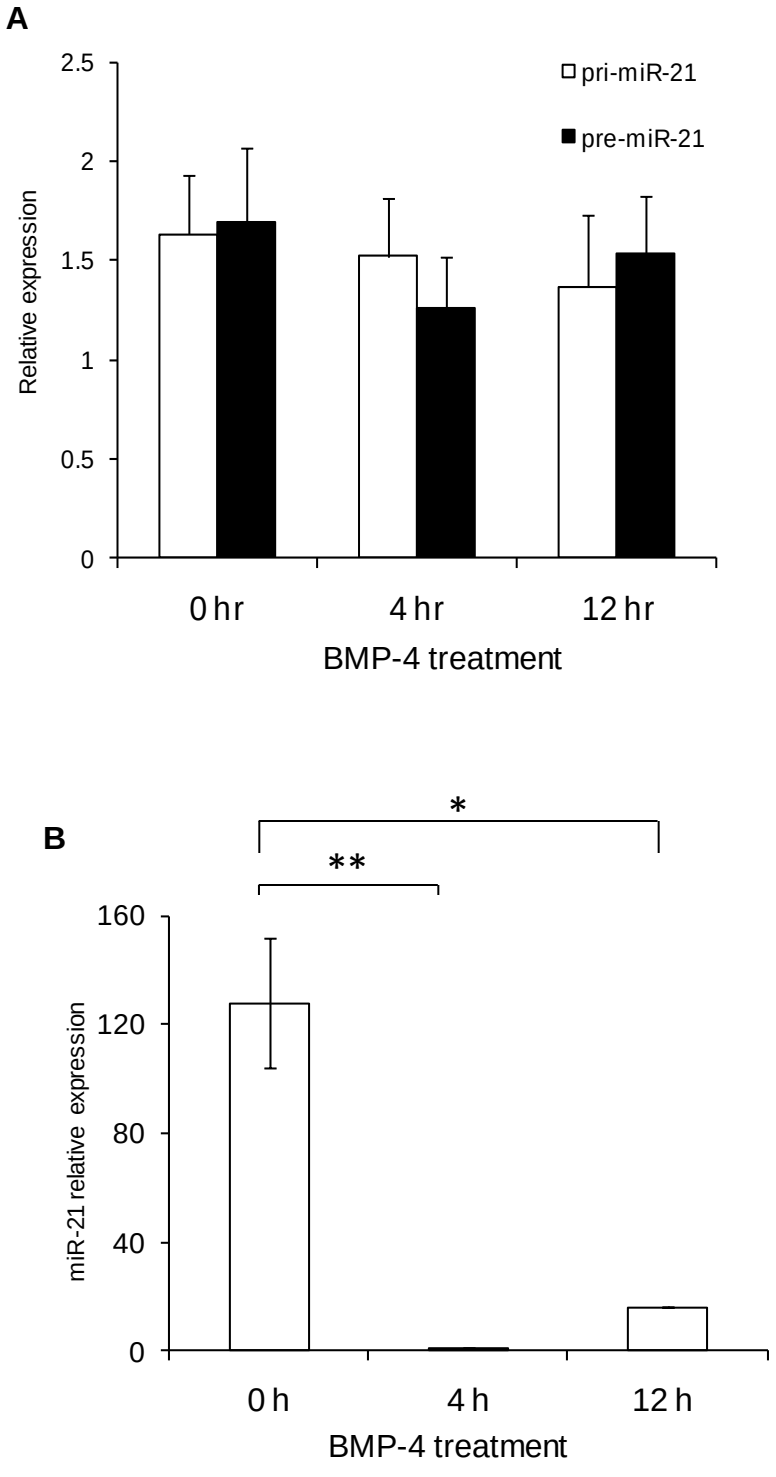


Figure 21. BMP4 inhibits expression of mature miR-21. A) By qRT-PCR transcript levels of primary-miR-21 and precursor-miR-21 after BMP4 treatment were unchanged**. B**) BMP4 treatment significantly reduces expression of mature form of miR-21 overtime. *P < 0.05, **P < 0.02. Unpaired student's t-test. n= 3.

3.3.2 MicroRNA-21 differentially regulates BMP target genes in primary mouse epidermal keratinocytes

To delineate the relationship between BMP-4 and miR-21 regulation of genes expression, PMEKs were treated with BMP4 and miR-21 mimic (pro-miR-21), and total RNA was analysed for changes in the expression of the genes that are i) known to mediate the effects of BMP, and ii) were experimentally proven to be a direct targets of miR-21. *Id1*, *Id2 Id3*, *Smad7* and *Msx2* genes were selected as known BMP target genes (Ogata *et al.,* 1993; Hussein *et al.,* 2003). Based on previous publications, *TPM1*, *Pdcd4*, *TIMP3* and *Pten* were chosen as the direct targets of miR-21 (Zhu *et al.,* 2007; Asangani *et al.,* 2008; Zhang *et al.,* 2008; Song *et al.,* 2010).

Treatment of the keratinocytes with BMP4 for 4 and 12 hours, resulted in the marked increase in mRNA levels of target genes of both BMP and miR-21, compared to the control cells (**Fig. 22*A,C***). In contrast, the expression of these genes was differently regulated in the keratinocytes by over-expressing miR-21. The levels of transcripts of BMP signalling target genes (*Id1*, *Id2 Id3*, and *Msx2*) remained unchanged in the keratinocytes transfected with pro-miR-21 (**Fig. 22*B***). However, the treatment with pro-miR-21 resulted in downregulation of the expression of *Pten*, *Pdcd4*, *TPM1*, and *TIMP3*, compared to the control cells (**Fig. 22*D***). The next step was to analyse, if miR-21 overexpression would abrogate BMP4-induced gene expression. To answer this question, keratinocytes were first

transfected with pro-miR-21 and then treated with BMP4. Pre-treatment of the keratinocytes with pro-miR-21 prevented the increase in mRNA levels of *Pten*, *Pdcd4*, *TPM1*, and *TIMP3* caused by BMP4 (**Fig. 23*A***).

In addition, the effect of miR-21 on Smad7 expression in keratinocytes was analysed. Smad7 is an inhibitory Smad protein, which antagonises TGF-β and BMP signalling (Hanyu *et al.,* 2001). Bioinformatic analysis of TargetScan and RNA22 revealed Smad7 carries two putative miR-21 binding sites (**Fig. 23*B***). In our experiments, BMP4 treatment significantly ($p<0.01$) increased the expression of Smad7 transcripts in the keratinocytes versus the control. However, Smad7 expression was significantly ($p<0.05$) reduced in the cells transfected with pro-miR-21 versus the control (**Fig. 22*C,D***). In addition, pre-treatment of the keratinocytes with pro-miR-21 prevented BMP4-induced Smad7 expression (**Fig. 23*A***). To validate whether miR-21 directly regulates expression of Smad7, the effects of miR-21 on Smad7 3'UTR was tested using a luciferase reporter assay.

Two predicted putative binding sites within the 3'UTR of *Smad7* mRNA were separately introduced into a luciferase reporter gene caused different effects on luciferase activity (**Fig. 23*C,D***). The first putative binding site within the 3'UTR of *Smad7* mRNA introduced into the luciferase reporter construct transfected with pro-miR-21, showed no effect on luciferase activity (**Fig. 23*C***). In contrast, co-transfection of HaCaT cells with the Smad7 3'UTR construct encompassing the second putative binding site, showed more than 60% reduction in luciferase activity

(**Fig. 23*D***). To assess whether this reduction of luminescence was specific, transfection of Smad7 3'UTR construct (binding site 2) was carried out with alternative miRNA, miR-31, which does not have any putative binding sites for Smad7. No effect on luciferase reporter activity was observed by miR-31 (**Fig. 23*D***)**.** Thus, Smad7 is a genuine target of miR-21.

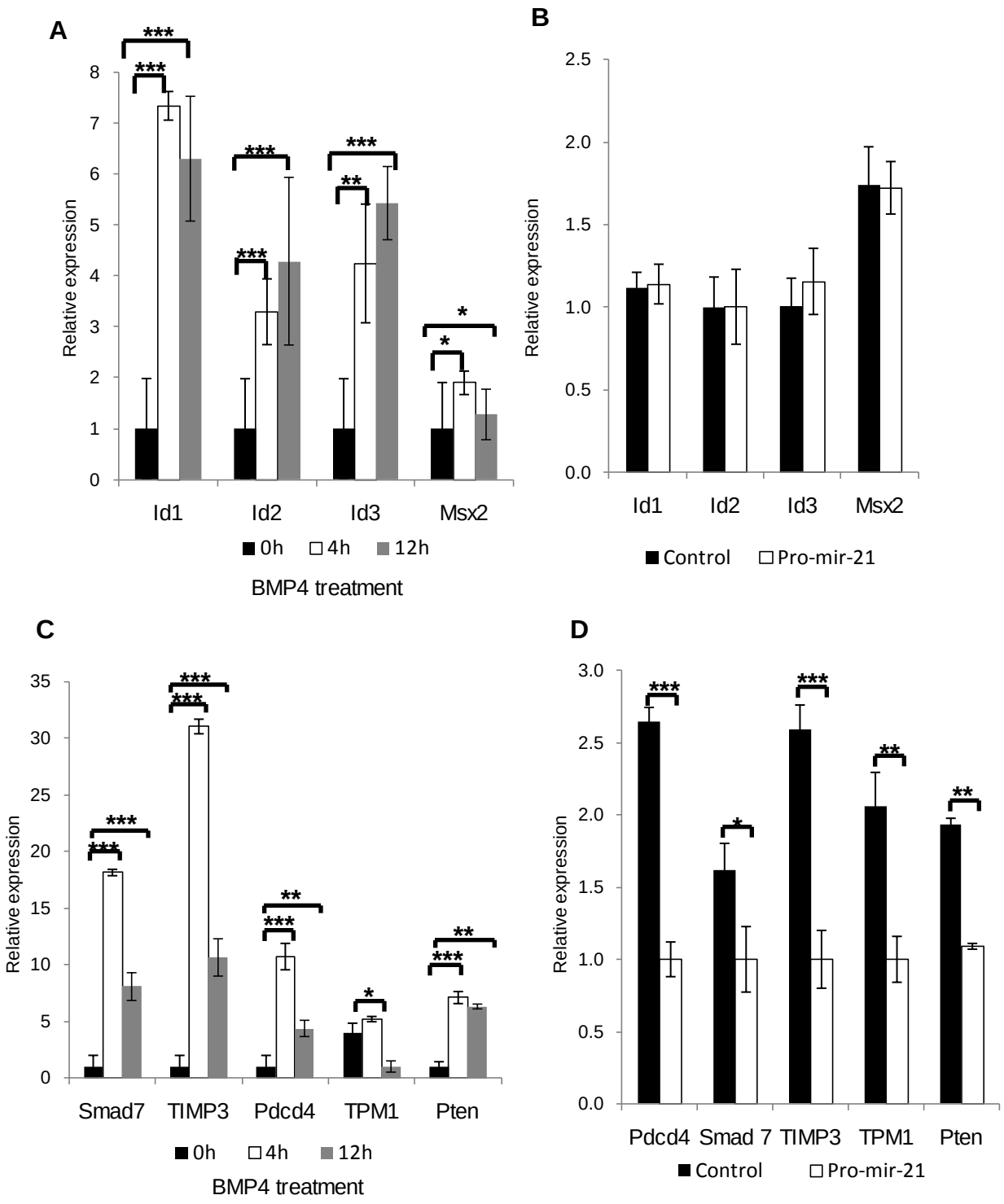
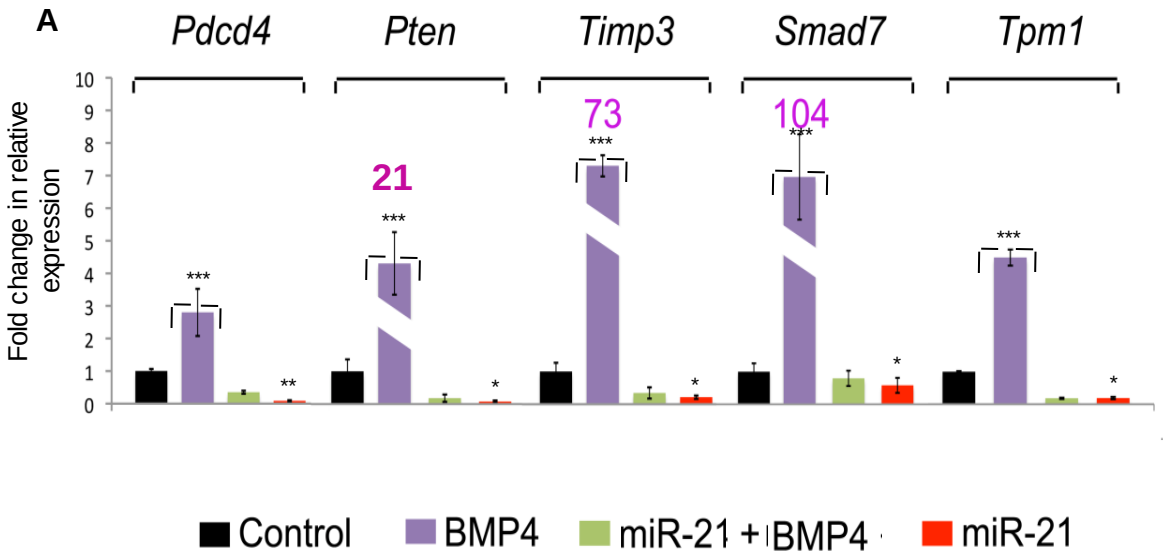
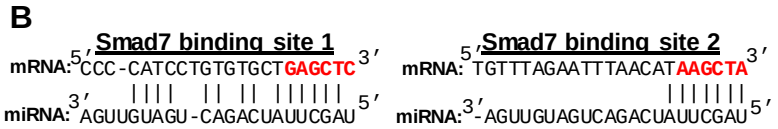
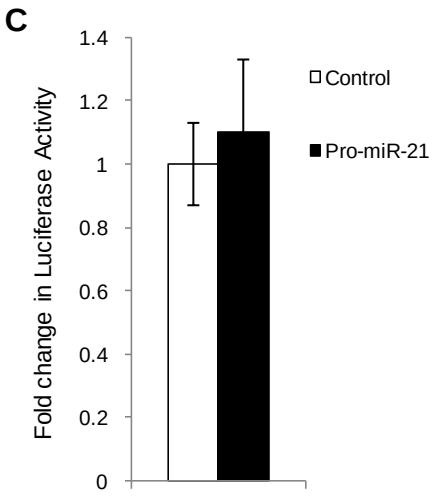
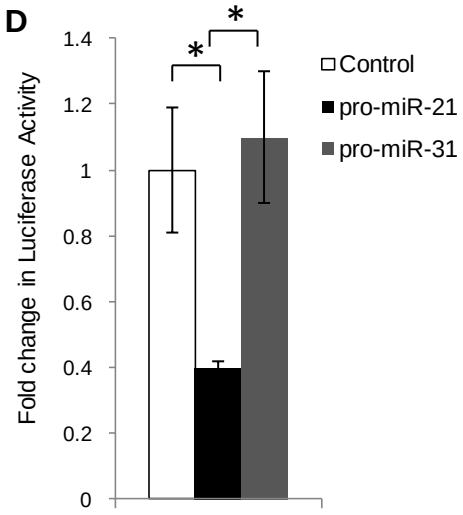

A
Relative expression
Id1
Id2
Id3
Msx2
0h
4h
12h
BMP4 treatment
B
Relative expression
Id1
Id2
Id3
Msx2
Control
Pro-mir-21
C
Relative expression
Smad7
TIMP3
Pdcd4
TPM1
Pten
0h
4h
12h
BMP4 treatment
D
Relative expression
Pdcd4
Smad 7
TIMP3
TPM1
Pten
Control
Pro-mir-21

Figure 22. Correlation in the regulation of gene expression between BMP4 and miR-21 using qRT-PCR. **A**) BMP4 treatment in keratinocytes resulted in significant increase in expression of Id1-3 and Msx2 compared to untreated cells. **B**) The expression of Id1-3 were not effected by miR-21 transfection compared with controls. **C**) Expression of Smad7, TIMP3, Pdcd4, TPM1 and Pten were upregulated after BMP4 treatment, with the most significant elevation in expression being detected after 4 hours. **D**) Expression of Smad7, TIMP3, Pdcd4, TPM1 and Pten in keratinocytes was reduced by miR-21 transfection compared with the controls. *$P < 0.05$, **$P < 0.02$, ***$P < 0.01$. Unpaired student's *t*-test . $n = 3$.


A
Pdcd4
Pten
Timp3
Smad7
Tpm1
Fold change in relative expression
21
73
104
Control
BMP4
miR-21 + BMP4
miR-21


B
Smad7 binding site 1
mRNA: 5′ CCC-CATCCTGTGTGCTGAGCTC 3′
miRNA: 3′ AGUUGUAGU-CAGACUAUUCGAU 5′
Smad7 binding site 2
mRNA: 5′ TGTTTAGAATTTAACATAAGCTA 3′
miRNA: 3′ -AGUUGUAGUCAGACUAUUCGAU 5′


C
Fold change in Luciferase Activity
Control
Pro-miR-21


D
Fold change in Luciferase Activity
Control
pro-miR-21
pro-miR-31

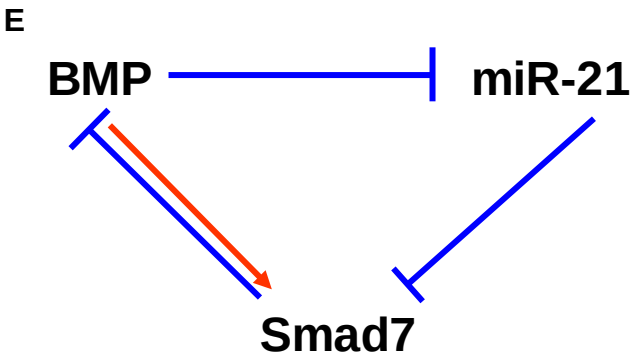

E
BMP
miR-21
Smad7

Figure 23. Correlation in the regulation of gene expression between BMP4 and miR-21. **A**) Expression of Smad7, TIMP3, Pdcd4, TPM1 and Pten was upregulated after BMP4 treatment, while transfection with pro-miR-21 prior to BMP4 exposure leads to dramatic reduction in the expression of Smad7, TIMP3, Pdcd4, TPM1 and Pten. **B**) Predicted interactions between miR-21 and *Smad7* mRNA. Alignment of mouse sequences in the 3'-UTR of *Smad7* mRNA. The seed region is shown in red. The representation is limited to the region around the miR-21 complementary site. **C**) The first putative binding site within the 3'UTR of *Smad7* mRNA introduced into the luciferase reporter construct showed no effect on luciferase activity. In contrast, co-transfection of HaCaT cells with the Smad7 3'UTR construct encompassing the second putative binding site showed more than 50% reduction in luciferase activity, compared to the corresponding control and miR-31. **E**) A schematic representation of the possible mechanism involved in the regulation of inhibitory Smad7 by BMPs via miR-21 in keratinocytes. $*P < 0.05$, $**P < 0.02$, $***P < 0.01$. Unpaired student's *t*-test . $n = 3$.

3.3.3 MicroRNA-21 expression is elevated in the skin tumours of mice over-expressing BMP inhibitor noggin

Recent reports have identified that miR-21 is a unique miRNA consistently overexpressed in tumours, including SCC of the skin (Si *et al.,* 2007; Selcuklu *et al.,* 2009; Dziunycz *et al.,* 2010). However, spatiotemporal expression of miR-21 in the skin has not yet been elucidated. Expression analysis of miR-21 was carried out in the skin of transgenic (TG) mice overexpressing the BMP antagonist noggin (under K14 promoter). These mice are characterized by spontaneous development of HF-derived tumours, which resemble human trichofolliculoma (Sharov *et al,* 2009).

K14-noggin mice showed significantly ($p<0.02$) higher levels of miR-21 transcripts, compared to the corresponding wild type mice (**Fig. 24*A***). By *in situ* hybridisation, weak miR-21 expression was observed in the epidermis and HF epithelium in anagen skin of control mice (**Fig. 24*B***). In K14-noggin mice, prominent miR-21 expression was seen in the tumours, preferentially in the peripheral portions (**Fig. 24*C***). It was previously shown the peripheral portions of the tumours in K14-noggin TG mice contain proliferating cells (Sharov *et al.,* 2009), which suggest that miR-21 can be involved in sustaining of the population of proliferative cells in the tumours.

Next, we assessed if expression of miR-21 target genes was changed in K14-noggin mice by qRT-PCR. Expression of *Pten*, *Pdcd4*, *TPM1*, *Smad7*, and *TIMP3*

transcripts were significantly decreased ($p<0.01$, $p<0.01$, $p<0.01$, $p<0.02$ and $p<0.02$, respectively) in K14-noggin mice, compared to wild type mice (**Fig. 24*D-F***). Therefore, these data demonstrates concurrent expression of miR-21 and its targets in the skin with inactive BMP signalling.

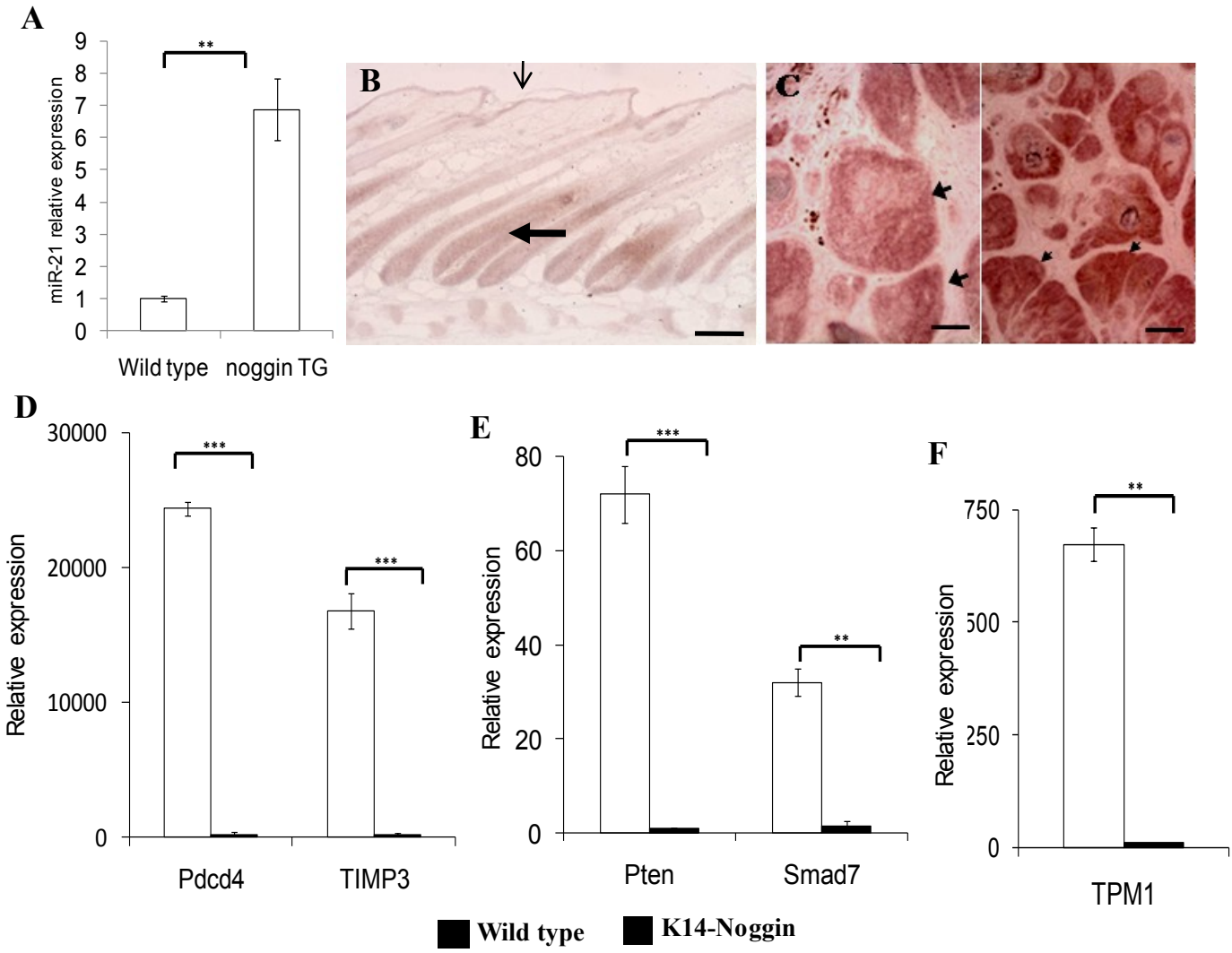


Figure 24. Increased expression of miR-21 in the skin of K14-Noggin mice. **A**) Detection of miR-21 in the skin of wild type and K14-noggin mice was carried out by qRT-PCR: miR-21 expression was elevated in the skin of K14-noggin mice compared with wild type. **B,C**) Representative microphotographs of *in situ* hybridization for miR-21 expression in skin of wild type and K14-noggin mice. **B**) Weak miR-21 expression was observed in the epidermis (small arrow) and HF epithelium (large arrow) in mouse anagen VI skin. **C**) In the skin of K14-noggin mice, prominent miR-21 expression was seen in the peripheral portion of the tumours (arrows). **D-F**) Analysis of miR-21-target genes by qRT-PCR: significant reduction in expression was observed for *Pdcd4*, *TIMP3*, *Pten*, *Smad7* and *TPM1* in the skin of K14-noggin mice compared to wild type mice. $^{**}P < 0.02$, $^{***}P < 0.01$. Unpaired student's *t*-test. *n*= 3. Scale bar 100μm.

3.3.4 MicroRNA-21 expression is elevated during chemically-induced carcinogenesis in murine skin

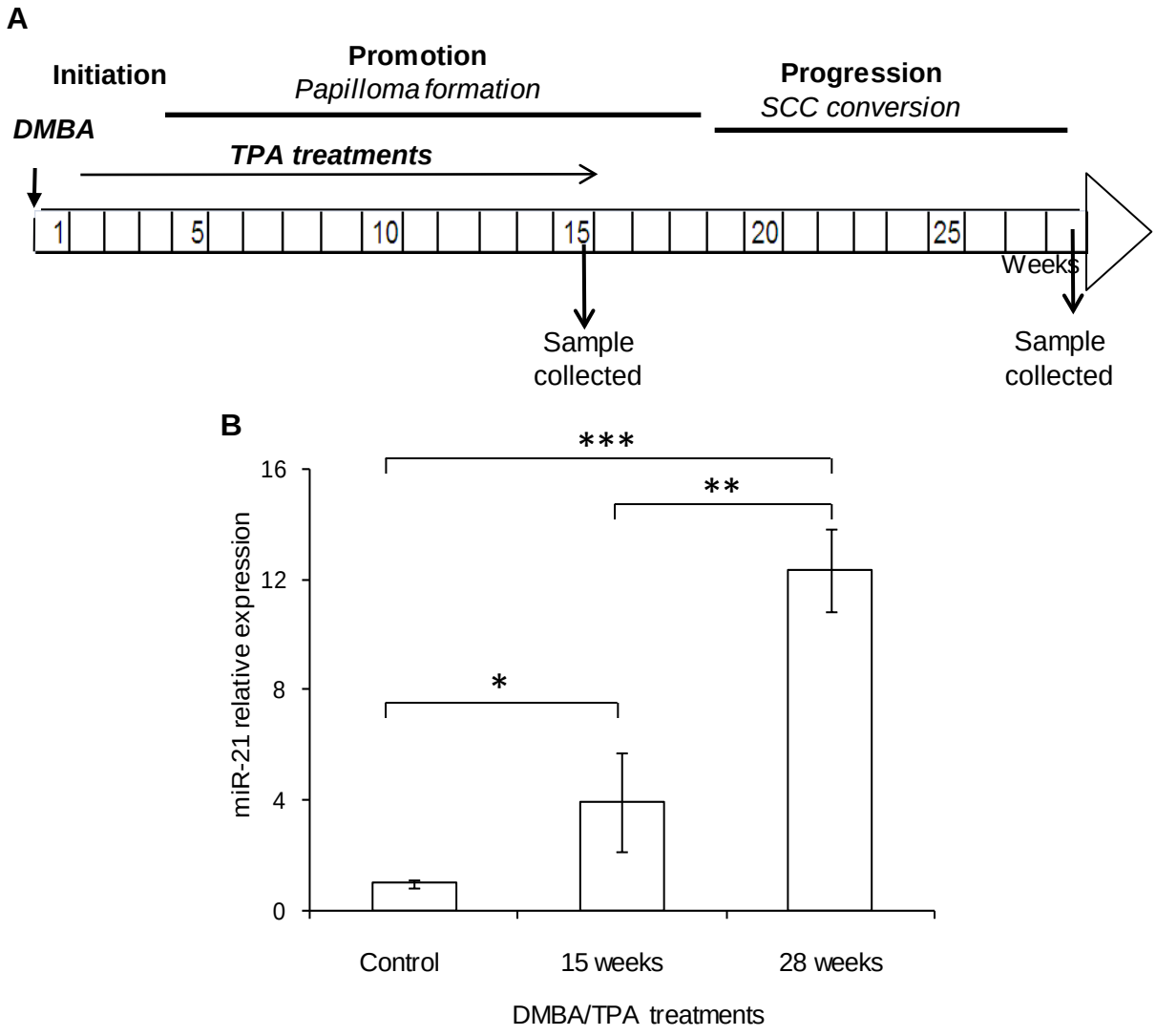
To define spatio-temporal expression of miR-21 at different stages of chemically-induced carcinogenesis, we utilized a two-stage chemical carcinogenesis procedure on mouse back skin involving DMBA/TPA treatments (DiGiovanni, 1992, **Fig. 25*A***).

Real-time PCR analysis revealed that in the skin treated with TPA for 15 weeks, miR-21 expression was significantly increased ($p<0.05$), compared to control mice (zero weeks of treatment). In the skin collected 28 weeks after the beginning of the experiment showed a further significant increase in the levels of miR-21 transcripts, compared to control (untreated) skin and in the skin treated with TPA for 15 weeks ($p<0.01$ and $p<0.02$, respectively) (**Fig. 25*B***).

By *in situ* hybridisation, very weak miR-21 expression was seen in the epidermis and HF epithelium in the control skin (untreated) (**Fig. 25*C***). In the skin treated with TPA for 15 weeks, more prominent miR-21 expression was seen in the hyperplastic epidermis compared with control skin (**Fig. 25*D***). In the skin collected 28 week after the start of the experiment, abundant miR-21 expression was seen in the hyperplastic epidermis and peripheral portion of the tumours containing low differentiated, proliferative cells, in “finger like” premalignant papillomas (**Fig. 25*E***). These data suggest that miR-21 is potentially involved in promoting and mataining of tumour development in the skin.

Summary

BMP signalling negatively regulates miR-21 expression in epidermal keratinocytes. There are two groups of BMP target genes, which expression is differentially regulated by miR-21. BMP mediates the effects on distinct groups of its targets in a miR-21-dependent and miR-21-independent manner. MiR-21 may operate as a pro-oncogene in the epidermis and promote neoplastic transformation of epidermal keratinocytes and cancer progression via the targeting of distinct tumour-suppressor genes and signalling pathways.


A
Initiation
Promotion
Papilloma formation
Progression
SCC conversion
DMBA
TPA treatments
1
5
10
15
20
25
Weeks
Sample collected
Sample collected
B
***
**
*
16
12
8
4
0
miR-21 relative expression
Control
15 weeks
28 weeks
DMBA/TPA treatments

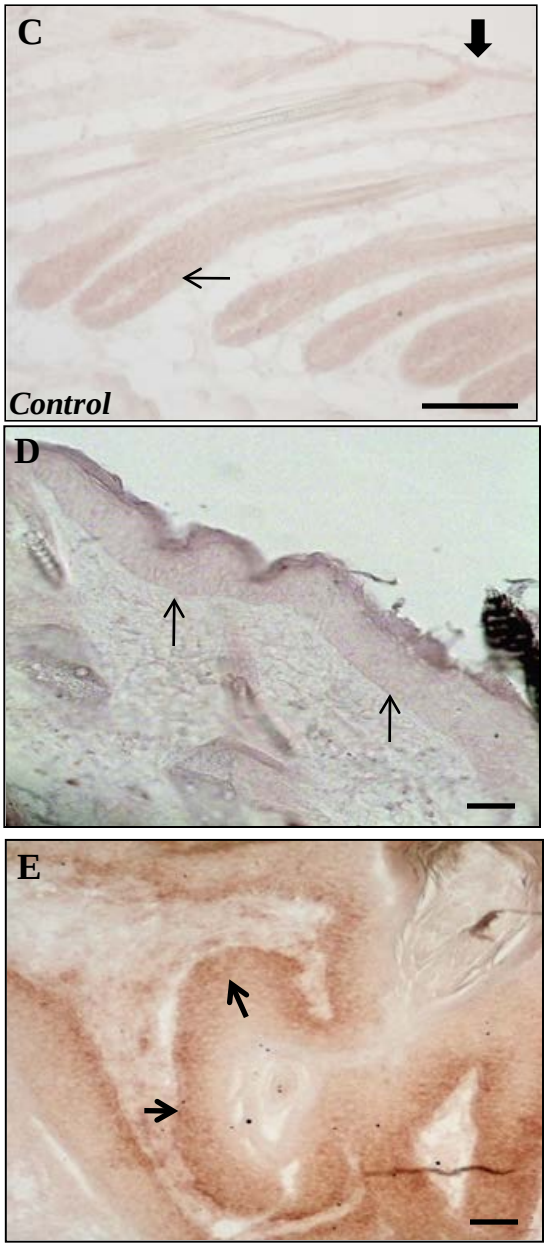

C
Control
D
E

Figure 25. Expression of miR-21 in skin during chemically induced carcinogenesis. A) A diagram showing DMBA/TPA protocol to induce multi-stage carcinogenesis in mouse back skin. Twice weekly applications of tumour promoter TPA was applied to mouse back skin for a total of 15 weeks. Carcinogenesis in mice can be divided into three discrete steps: i) initiation, ii) promotion (papillomas formation) and iii) malignant conversion (SCC). Skin samples were collected at 15 weeks and 28 weeks after the beginning of the experiment. **B**) Detection of miR-21 expression in the skin during chemically induced carcinogenesis was carried out by qRT-PCR: in the skin treated with TPA for 15 weeks, miR-21 expression was upregulated compared with control (untreated skin). In skin sample collected 28 weeks after the beginning of the experiment, miR-21 expression increased further compared with samples collected 15 weeks after treatment and control (untreated skin). **C-E**) Representative microphotographs of *in situ* hybridization for miR-21 in skin during chemically induced carcinogenesis. **C**) In control skin, very weak miR-21 expression was detected in the HF epithelium (small arrow) and epidermis (large arrow) **D**) In skin treated with TPA for 15 weeks, miR-21 expression was detected in the hyperplastic epidermis (arrows). **E**) In skin collected 28 weeks after the beginning of the experiment, abundant miR-21 expression was detected in the peripheral portion of the tumours (arrows). *P <0.05, ***P* <0.02, ****P* <0.01. Unpaired student's *t*-test. *n*= 3. Scale bar 100μm. Abbreviations: DMBA, dimethylbenz[a]antracen; TPA, 12-tetradecanoil-phorbol-13-acetate.

Chapter IV

DISCUSSION

4.1 Complex changes in miRNA expression during distinct stages of hair follicle cycle

HF cycling is a unique biological phenomenon that is accompanied by the profound changes in the skin and HF microanatomy and in pigmentation, as well as by the remodelling of the cutaneous vascular and nervous apparatus (Botchkarev *et al.,* 1999; Mecklenburg et al., 2000; Peters *et al.,* 2001; Stenn and Paus, 2001; Slominski *et al.,* 2004; Slominski *et al.,* 2005). This study revealed that each stage of the hair cycle is characterized by the distinct patterns of the miRNA profiles in the skin (**Fig. 6**), thus suggesting miRNAs as an important regulatory layer in the complex program controlling hair cycle-associated changes of gene expression in the skin and the HF.

Remarkable fluctuations in the expression levels of distinct miRNAs during hair cycle suggest that these miRNAs may be involved in at least 3 distinct mechanisms of the control of gene expression proposed for miRNAs previously: 1) silencing of the distinct gene expression programs during the HF transition between hair cycle stages (i.e., switch between the telogen associated growth inhibitory programs and hair growth promoting programs during the telogen-anagen transition or between the hair growth-associated programs to pro-apoptotic programs during transition of the HFs from anagen to catagen); 2) establishing the optimal levels of the transcripts to prevent their overexpression, thus modulating a balance of activity of distinct signalling pathways (i.e., limiting an excessive activation of pro-carcinogenic signalling pathways, such as Wnt or Shh, during active HF growth and hair

production; and 3) control of the expression of the other miRNAs to limit their inhibitory effects on the levels of the target transcripts (i.e., similar to the antagonistic interactions between the miR-184 and miR-205 described previously (Yu *et al.,* 2008).

4.1.1 MicroRNA-31 in complex regulation of the gene expression programs that control anagen progression and hair shaft formation in the HF

Although the function of the distinct miRNAs or their clusters in the control of HF cycling remains to be further defined, here evidence is provided that miR-31 plays an important role in the control of anagen-associated gene expression programs in the HF.

MiR-31 expression is markedly increased in the skin during anagen phase of the hair cycle, and its expression is seen in both the epidermal and follicular epithelial skin compartments, as well as in the DP (**Fig. 7**). Furthermore, inhibition of the miR-31 activity in the skin results in anagen acceleration and in alterations in the hair shaft formation and ORS morphology (**Fig. 8, Fig. 9**).

However, changes in miR-31 expression levels were observed using either anti-miR-31 or miR-31 mimic did not affect proliferation *in vitro* (**Fig. 10**). These findings are consistent with recently reported data showing that overexpression of miR-31 in aggressive breast cancer cells does not affect their proliferation when compared to normal mammary epithelial cells (Valastyan *et al.,* 2009). This suggests that elevated expression of miR-31 in cells with high proliferative activity is possibly

required to prevent alterations in growth factor signalling leading to tumour initiation or progression.

In addition, these data show the inhibitory effects of miR-31 on anagen development are likely to be realized, at least in part, via modulation of the activity of the Fgf, Wnt, and BMP signalling pathways. By analyzing the global gene expression changes in primary keratinocytes treated with anti-miR-31, demonstrated that miR-31 is involved in regulating the expression of the several components of these pathways, such as the FGF receptor ligand Fgf10, the Wnt and BMP inhibitor Sost, and the BMP antagonist Bambi (**Table 4**).

Moreover, Fgf10 might serve as one of the direct targets of miR-31 (**Fig. 14**). Fgf and Wnt pathways promote the HF telogen-anagen transition via providing the stimulatory signals to the HF stem cells and/or their progenies residing in the HF bulge and SHG, while BMP signalling operates as an anagen inhibitor antagonizing the activity of the Fgf and Wnt pathways during telogen (Botchkarev *et al.,* 2001a; Van Mater *et al.,* 2003; Plikus *et al.,* 2008; Greco *et al.,* 2009). Sost is capable of antagonizing the Wnt and BMP pathways in osteoblasts and adipocytes; however, it can also directly bind noggin, a potent BMP antagonist, stimulator of telogen-anagen transition and modulator of hair shaft pigmentation (Sharov *et al*., 2005; Winkler *et al.,* 2004). Thus, by regulating the expression of the Fgf10, Bambi, and Sost miR-31 may be involved in the fine tuning the activity of the Fgf, Wnt, and BMP pathways in distinct subpopulations of hair progenitor cells and in modulating the effects of these pathways on anagen progression and hair shaft pigmentation associated with it.

However, in addition to the effects of miR-31 on early stages of anagen development, inhibition of the miR-31 activity during mid-/late anagen results in alterations in the hair shaft formation accompanied by its thickening, irregular distribution of the pigment, as well as by the hyperplasia of the ORS (**Fig. 9**). Fgf, Wnt, and BMP signalling pathways are closely involved in the control of keratinocyte differentiation and hair shaft formation, and genetic alterations in the activity of these pathways results in the distinct abnormalities of the hair shaft formation and pigmentation (Schneider *et al.,* 2001; Alonso and Fuchs, 2006; Sharov *et al.,* 2006).

Evidence is provided here that, in addition, to the modulatory effects on the activity of the Fgf, Wnt, and BMP pathways, miR-31 is also involved in the control of hair shaft formation, at least in part, via regulating the expression of the Dlx3 transcription factor. Expression of the Dlx3 in the skin samples treated with anti-miR-31 was significantly upregulated and, in addition to the expression in the hair matrix, IRS and hair shaft, Dlx3 is ectopically expressed in the ORS (**Fig. 13**). The luciferase reporter assay confirmed that expression of Dlx3 is directly regulated by miR-31 (**Fig. 14*B***).

Dlx3 transcription factor plays an important role in the control of the hair matrix keratinocyte differentiation toward the hair shaft and IRS cell lineages. Genetic Dlx3 ablation in mice results in failure of proper formation of the hair shaft and IRS leading to complete alopecia (Hwang *et al.,* 2008). Dlx3 also serves as a downstream target of the Wnt and BMP signalling pathways and as an upstream regulator of the Hoxc13 and GATA-3 transcription factors that are essential

components of the transcription programs controlling the formation of the hair shaft and IRS, respectively (Jave-Suarez *et al.,* 2002; Kaufman *et al.,* 2003; Kurek *et al.,* 2007).

Thus, elevated Dlx3 expression and its ectopic appearance in the ORS in response to miR-31 inhibition (**Fig. 13*E,F***) could contribute to the abnormalities in the hair shaft and ORS morphology seen in this study (**Fig. 9**). In addition to the changes observed in the expression of distinct signalling molecules, we demonstrate here that miR-31 regulates the expression of the K14, K16, and K17, the essential components of the keratinocyte cytoskeleton. Alterations in the levels of miR-31 induced by miR-31 synthetic inhibitor resulted in up-regulation of the expression of these keratins both *in vitro* and *in vivo* (**Fig. 12**). Moreover, luciferase reporter assay revealed that expression of K16 and K17 are directly regulated by miR-31 through their 3'UTR, whereas K14 are most likely an indirect target of miR-31 (**Fig. 14**).

Alterations of the hair shaft structure and ORS hyperplasia were found after inhibition of the miR-31 activity in the skin are accompanied by up-regulation of the expression of K17 in the hair matrix keratinocytes, hair shaft medulla, and ORS, as well as by increase of the K16 expression in the companion layer of the ORS (**Fig. 12*G-J***). In addition to the maintenance of keratinocyte cytoskeleton, K17 is capable of influencing keratinocyte growth and size by regulating protein synthesis (Kim *et al.,* 2006). This suggests that K17 may contribute, at least in part, to the acceleration of anagen development and hyperplastic changes in the ORS in the HF treated by miR-31.

K16 and K17 are expressed in the medulla of the central portion of the hair shaft (McGowen *et al.,* 1998; Bernot *et al.,* 2002). K16 overexpression under control of the K14 promoter leads to the formation of curly hairs and hyperplasia of the ORS (Paladini and Coulombe, 1998). Genetic ablation of the keratin 17 results in hair shaft fragility and strain-dependent alopecia (McGowen *et al.,* 2002), while intradermal administration of K17 oligonucleotides leads to marked alterations of the hair shaft morphology and shape (Fan and Yoon, 2003). These data suggest that the elevated levels of the K16 and K17 may, at least in part, contribute to the alterations in the hair shaft structure seen after anti-miR-31 treatment.

4.2 A role of microRNA-214 in regulating the hair follicle cycle

Knowledge of the biological mechanisms regulating mammalian development is highly important for better understanding of the changes occurring in health and disease. This is evident from the fact that many signalling pathways that control organ development such as: Wnt/β-catenin signalling, *Shh*, TGF-β/BMPs, Notch, FGFs and so on, are also involved in postnatal organ growth and regeneration (Fuchs, 1998; Schmidt-Ullrich and Paus, 2005). Moreover, deregulation of any of these signalling pathways can lead to alteration in cell proliferation, differentiation, and apoptosis in many tissues, including the skin, resulting in impaired tissue repair and cancer development (Gat *et al.,* 1998; Makrantonaki and Zouboulis, 2007; Nicolas *et al.,* 2003; Farage *et al.,* 2009; Mimeault and Batra, 2009).

The role of miRNAs in regulating cellular processes such as cell proliferation, differentiation and programmed cell death has been shown in many tissues including the skin. MiR-214 expression was analysed in cancer stem cell differentiation, where it was shown miR-214 along with miR-199a could regulate cell differentiation of epithelial ovarian cancer stem cells (Yin *et al.,* 2010). In addition, in zebra fish, miR-214 was shown to have negative effects on Shh signalling (Flynt *et al.,* 2007). The regulatory effects of miR-214 on Shh signalling in zebra fish could be of evolutionary importance and potentially conserved in mammals. This regulatory mechanism maybe involved in regulating skin homeostasis. However, in mammals Shh has not been predicted as a target of miR-214. In contrast, β-catenin was bioinformatically

identified in this study as an evolutionary conserved target for miR-214. β-catenin is one the key components of Wnt signalling pathway, which is one of the most crucial signalling pathways in mammalian development. In the skin, it has been shown to be vital in the formation of hair placodes, hair shaft differentiation, and maintenance of skin stem cells (Gat *et al.,* 1998; Huelsken *et al.,* 2001). Deregulation in Wnt signalling pathway can lead to skin disorder such as, psoriasis (Hampton *et al.,* 2007) and some epithelial tumours (Kinzler *et al.,* 1991; Nishisho *et al.,* 1991; Chan *et al.,* 1999; Gat *et al.,* 1998; Tetsu and McCormick, 1999; Jamieson *et al.,* 2004; Celso *et al.,* 2004). This project attempted to elucidate the role of miR-214 and its regulatory role on Wnt/β-catenin in HF development.

Global miRNA expression profiling validated by qRT-PCR, showed elevated levels of miR-214 transcripts in neonatal skin during the anagen-like stage at P12, compared with low expression in catagen and telogen stages (**Fig. 15**). *In situ* hybridization showed that miR-214 is predominantly expressed in the HF SHG and bulge regions in telogen skin (**Fig. 16*A***). The importance of Wnt/β-catenin signalling in telogen-anagen transition was indentified from several studies. Data obtained from TOPgal transgenic mice carrying a Lef/TCF-responsive-element controlled reporter gene *LacZ*, showed an increase in β-galactosidase in the bulge region of the HFs at the onset of anagen (DasGupta and Fuchs, 1999). Furthermore, Wnt transcripts and the level of nuclear β-catenin accumulation have been shown to be elevated during late telogen/early anagen (Greco *et al.,* 2008), suggesting that the stabilization of β-catenin, a transcriptional cofactor for Lef1/TCF proteins is required for the activation

of stem cells in bulge region leading to the onset of new hair growth cycle (Gat *et al.,* 1998; Celso *et al.,* 2004; Lowry *et al.,* 2005; Van Mater *et al.,* 2003). Contrasting patterns in miR-214 expression and Wnt-dependent β-catenin stabilization suggests that the latter maybe in part regulated by miR-214.

Telogen stage is governed by a balance of growth activators and inhibitors. Alterations in this balance results in either initiation or prevention of active growth phase. In this context, the expression of miR-214 in the bulge region of the resting HFs may be involved in regulating the onset of anagen during the HF cycle through the regulation of β-catenin. Moreover, miR-214 may provide an additional regulatory mechanism in maintaining stem cell quiescence during telogen.

During mid-anagen, miR-214 expression remained in the epidermis and HF bulge, as well as appeared in the HF bulb, ORS and DP (**Fig. 16*B***). However, expression was absent from the pre-cortex region of the HF. The absence of miR-214 expression in the pre-cortex correlates with the study, showing elevated Lef-1 and nuclear β-catenin expression during pre-cortex formation in matrix cells (DasGupta and Fuchs, 1999). Elevated Lef-1 expression in the matrix cells has shown to be essential for commitment of keratinocytes to hair shaft differentiation (DasGupta and Fuchs, 1999). Furthermore, Lef-1 knockout mice show arrest of HF development prior to the beginning of hair shaft formation (Van Genderen *et al.,* 1994; DasGupta and Fuchs, 1999). This suggests that miR-214 may regulate Wnt/β-catenin/Lef-1

signalling involvement in the conversion of proliferating keratinocytes to hair shaft forming cells.

In late anagen HFs, miR-214 was prominently expressed in the hair matrix, IRS and ORS (**Fig. 16*C***), while in catagen, its expression gradually decreased, being mainly restricted to regressing HF matrix (**Fig. 16*D***). In contrast, it was shown previously that no Wnt activity was detected in catagen and telogen stages (DasGupta and Fuchs, 1999).

Taken together, analysis of miR-214 expression suggests that its expression might be tightly associated with Wnt activity during distinct stages of the HF cycle, and contributes to Wnt/β-catenin signalling dependent changes in cell fate and differentiation commitments which take place. This study highlights miR-214 as a potential regulator of maintaining the balance of Wnt/β-catenin pathway activity by preventing its excessive activation in the keratinocytes at different stages of postnatal HF remodelling.

4.2.1 A role for microRNA-214 in regulating Wnt signalling pathway during hair follicle morphogenesis

As the next approach to validate our hypothesis that miR-214 is involved in the control of Wnt-β-catenin signalling, analysis of the expression and potential co-localisation of miR-214 and β-catenin during HF morphogenesis was performed. Our data revealed mutual exclusive expression of miR-214 and β-catenin during HF development (**Fig. 17**). The Wnt/β-catenin/Lef-1 signalling pathway was shown to be one of the crucial pathways involved in initiating HF development. Overexpression of a Wnt-antagonist, Dkk-1, in murine epidermis was shown to lead in the abrogation of induction of all HFs (Andl *et al.,* 2002), while overexpression of β-catenin results in *de novo* HF formation (Gat *et al.,* 1998). The presence of miR-214 at sites of Wnt/β-catenin activity suggests that miR-214 may determine HF induction by regulating Wnt activity in the skin. In HFs at advanced stages of HF morphogenesis, miR-214 expression was initially detected in the peripheral regions of the HF (stage 5), while expression of β-catenin was observed in the central differentiating keratinocytes of the growing HFs. MiR-214 expression reduced in more advanced HFs (stage 6), while β-catenin expression reduced in the epidermis but increased in the stalk of these HFs. β-catenin expression in the epidermis and HF correlates with current knowledge that *Lef1* mRNA expression is weaker in the basal layer of epidermis, but increases in the hair bulb, in epithelial cells destined to express HF-specific keratins genes during HF morphogenesis (Zhou *et al.,* 1995; Gat *et al.,* 1998; DasGupta and Fuchs, 1999). This mutual exclusive expression pattern of miR-214 and β-catenin

suggests miR-214 as a possible negative regulator of Wnt signalling via targeting β-catenin during HF development. This negative regulation maybe involved in maintaining balance of hair keratinocyte proliferation/differentiation and thus may regulate skin and HF morphogenesis.

4.2.2 MicroRNA-214 regulates the activity of the Wnt/β-catenin signalling pathway in keratinocytes

To test whether the spatiotemporal expression pattern of miR-214 has indeed a functional link with Wnt/β-catenin pathway, the effects of miR-214 on the activity of β-catenin in keratinocytes was analysed. Transfection of keratinocytes with synthetic miR-214 mimic resulted in reduced levels of β-catenin transcripts and protein (**Fig. 18*A-C***). Furthermore, miR-214 prevented translocation of β-catenin into the nucleus in response to the treatment with an activator of the Wnt signalling pathway lithium chloride, and abrogated the lithium chloride-induced increase of the expression of the Wnt target gene *Axin2*. In addition, analysis of the effects of miR-214 on TOPflash plasmid construct showed that activation of the Wnt-responsive element abrogated by miR-214. Moreover, the luciferase reporter assay confirmed that expression of β-catenin is directly regulated by miR-214 (**Fig. 18*E***) and therefore, β-catenin is indeed a genuine target of miR-214. Thus, these data demonstrate that miR-214 can be involved in the regulation of the activity of the Wnt/β-catenin signalling pathway in the keratinocytes.

These findings implicate miR-214 as a novel regulator of the Wnt/β-catenin signalling pathway and therefore, provides an additional regulatory mechanism of involvement of Wnt/β-catenin signalling in HF induction, stem cell activation/maintenance and hair shaft differentiation (Gat *et al.,* 1998; Huelsken *et al.,* 2001).

In addition, miR-214 negatively regulates Wnt targets such as c-myc and cyclin D1. The *c-myc* proto-oncogene encodes the c-myc transcription factor, and is broadly expressed during skin embryogenesis and in tissue compartments that possess high proliferative capacity (i.e., the basal layer of the epidermis). Sustained activation of c-myc in the skin has been shown to result in epidermal hyperplasia with areas of focal dysplasia leading to papillomatous lesions that resemble the human premalignant skin lesions known as actinic keratosis, a precursor of SCC (Pelengaris *et al.,* 2002). Most *in vitro* and *in vivo* studies have indicated that the predominant role of deregulated c-myc is to initiate/progress tumours via uncontrolled cell proliferation, reduced cell apoptosis and a loss of terminal differentiation in keratinocytes. In the HF, c-myc is expressed in the cells of anagen follicles, located in the IRS and ORS (Rumio *et al.,* 2000; Bull *et al.,* 2005). Activation of c-myc was shown to lead to elevated epithelial cell proliferation of ORS with reduced levels of apoptosis. Moreover, overexpression of c-myc resulted in marked desynchronization of the murine hair growth cycle, uncoupling of hair cycle-related skin thickness and hyperplasia of the ORS (Bull *et al.,* 2005).

Furthermore, cyclin D1 was detected in the bulge region in the telogen HF, which elevated during anagen onset and in anagen HFs it was detected in the ORS (Xu *et al.,* 2003). Constitutive activation of Wnt/β-catenin/Lef-1/cyclin-D1 signalling leads to uncontrolled progression of cell cycle, a characteristic of cancer development (Shtutman *et al.,* 1999; Xia *et al.,* 2001). These expression patterns of c-myc and cyclin D1, coincides with miR-214 expression in corresponding HF stages. This suggests miR-214 may regulate cell proliferation and differentiation of epithelial cells and prevent the development of epithelial tumours by negative regulation of β-catenin target genes in the skin and HF.

The data presented here suggests also that miR-214 negatively regulates expression of Pten. Interestingly, Pten is a known target of miR-214 as it was shown in human ovarian cancer cells (Yang *et al.,* 2008). In the HF, Pten is expressed in the bulge region and in the ORS of anagen HFs (Tsao *et al.,* 2003; Zhang *et al.,* 2006). Keratinocytes-specific mutations of Pten in mice have shown to lead to accelerated HF morphogenesis (Suzuki *et al.,* 2003). MiRNAs have been shown to have crucial regulatory roles in cell differentiation and proliferation and maintenance of skin stem cells. Therefore, miR-214 may represent a unique miRNA, which potentially can regulate skin and HF development and cycling, by targeting β-catenin and negatively regulating c-myc, cyclin D1, Axin2 and Pten, which are crucial players in skin and HF development, stem cell differentiation and carcinogenesis.

These data suggest that the role of miR-214 in the skin and HF may be first and foremost to prevent highly stable forms of β-catenin becoming constitutively active;

thus by regulating a crucial pathway such as the canonical Wnt pathway, it can potentially regulate a diverse range of cellular processes involved in skin and HF development and HF cycling and tumourigenesis.

4.3 BMP signalling mediate its effects in the skin in part through post-transcriptional regulation of miR-21

BMPs are multifunctional growth factors that induce biological effects through the BMP receptor complex followed by activation of the BMP-Smad or BMP-MAPK signal transduction pathways (Botchkarev, 2003). BMP signalling plays a pivotal role in regulating of many developmental programs and regeneration in numerous organs including the skin, by regulation of cell proliferation, differentiation, and apoptosis (Botchkarev and Paus 2003; Li, Greco *et al.,* 2003; Massague 2003; Mishina 2003; Plikus *et al.,* 2008).

BMPs have been shown to inhibit the initiation phase of the HF morphogenesis (Botchkarev *et al.,* 1999). In adult skin, BMP signalling acts as an inhibitor in telogen phase for hair cycle induction (Botchkarev, et al 2001), while at later stages it is required for proper control of keratinocyte differentiation into HF-specific cell lineages (Kobielak *et al.,* 2003; Andl *et al.,* 2004; Kin Ming *et al.,* 2004; Yuhki e*t al.,* 2004). BMP signalling also controls hair pigmentation by interaction with the melanocortin receptor-1 signalling to modulate the balance between pheomelanogenesis and eumelanogenesis during hair growth (Sharov *et al,* 2005).

Skin cancer including squamous and basal cell carcinoma, represent the most frequent type of malignancy in the Caucasian population and its incidence continues to grow in the UK and worldwide (Glick and Yuspa, 2005). Several studies have shown BMP signalling is important in regulating skin tumorigenesis. Overexpression

of BMP4/6 in murine epidermis was shown to lead to increased resistance to chemically induced carcinogenesis, while conditional ablation of the BMPR-IA in keratinocytes results in formation of HF-derived tumours (Ming-Kwan *et al.,* 2004). Anti-tumour activities of BMP signalling have been shown to be suppressed by extracellular BMP inhibitors such as gremlin 1 and noggin. Gremlin 1 expression was shown to be increased in basal cell carcinomas, in which gremlin promotes cell proliferation, in contrast to BMPs (Sneddon *et al.,* 2006). Recently, BMP antagonist noggin was shown to promote skin tumorigenesis via stimulation of the Wnt and Shh signalling pathways (Sharov *et al.,* 2009).

However, recent findings have suggested BMP tumour suppressive effects may be mediated by miRNAs (Reinke and Carthew, 2008). Increasing evidence suggests that miRNAs are important regulators of the oncogenic processes, and the expression of many miRNAs are deregulated in cancers (Deng *et al.,* 2008). By targeting multiple signalling pathways miRNAs can either potentially prevent cancer development or contribute to cancer initiation and progression (Selcuklu *et al.,* 2009; Inui *et al.,* 2010). In this project, analysis of the spatio-temporal expression of miR-21, which possesses oncogenic activity, was carried out in K14-noggin mice, to investigate whether some of these inhibitory effects of the BMP signalling are mediated in part by miRNAs.

Microarray and real-time PCR analyses revealed substantial BMP4-dependent changes in the expression of distinct miRNAs (**Fig. 20**). Among them, miR-21 expression was markedly decreased in keratinocytes after BMP4 treatment (**Table**

5). This was followed by analysis of the effects of BMP4 on miR-21 biogenesis, which showed BMP4 only negatively regulated mature form of miR-21. This suggests that BMP4 negatively regulates expression of miR-21 post-transcriptionally, involving only in the final step of its biogenesis (**Fig. 21**). In contrast, in the skin of TG mice overexpressing noggin, expression of miR-21 was increased compared to wild-type mice (**Fig. 24**). These data are also consistent with recent findings that BMPs can downregulate miR-21 in breast cancer. BMP-6-induced inhibition of miR-21 was suggested as a possible anti-metastasis factor by a mechanism involving post-transcriptional repression of miR-21 in breast cancer (Du *et al.,* 2009). Therefore, negative regulation of miR-21 by BMP signalling identified in this study might suggest a novel molecular mechanism for BMP-induced tumour suppressive effects in the skin.

Furthermore, we identified the existence of two groups of the BMP target genes, which are differentially regulated by miR-21. BMP-dependent tumour-suppressor genes such as *Pdcd4*, *Pten*, *TPM1*, and *Smad7*, are negatively regulated by miR-21, In fact, the downregulation of *Pten*, *Pdcd4*, *TPM1*, *TIMP3* expression in the keratinocytes due to their transfection with miR-21 mimic was anticipated, because these genes were experimentally proven to be the targets of miR-21 and have been shown to be either mutated or downregulated in many tumours (Zhu *et al.,* 2007; Asangani *et al.,* 2007; Li *et al.,* 2009; Zhang *et al.,* 2008; Song *et al.,* 2010). Therefore, this suggests further that the anti-tumour effects of BMP signalling in keratinocytes can indeed be mediated by miR-21. BMP signalling may acquire its

anti-tumour functions in the skin in part by down-regulating miR-21 expression, which will in turn abrogate negative effects of miR-21 on tumour suppressor genes. However, we identified that the expression of genes, which mediate BMP-induced cell differentiation effects, such as *Id1-3*, and *Msx2*, is not affected by miR-21. This suggests that BMP signalling pathway in keratinocytes mediates its diverse functions in miR-21-dependent and miR-21-independent manner (**Fig. 26**).

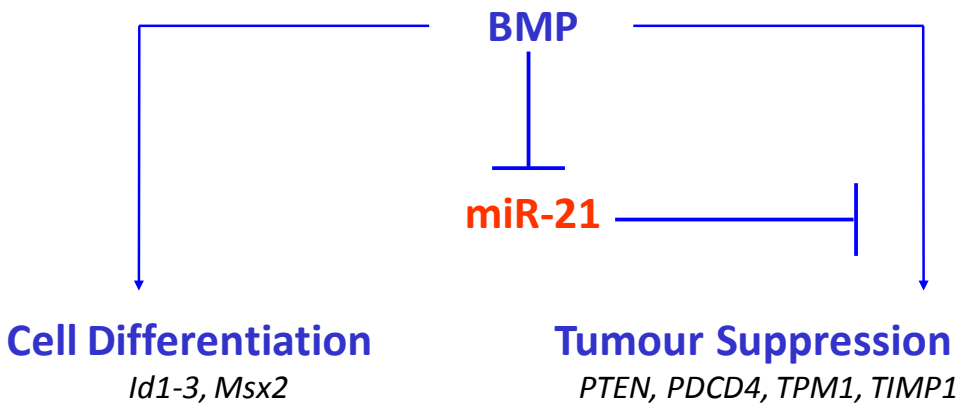


Figure 26. Illustrative representation of the complex regulatory pathways involved in BMP signalling in the skin. BMP signalling negatively regulates miR-21 expression in epidermal keratinocytes. There are two groups of BMP target genes, which expression is differentially regulated by miR-21.

In addition, this study revealed that Smad7 is a direct target of miR-21 (**Fig. 23**). Smad7 is an inhibitory protein, which mediates negative feedback loop to BMP signalling. It has been shown that inhibitory Smad7 not only antagonizes BMP signalling, but its expression is also induced by BMP signalling (Denissova *et al.*, 2000). The negative regulation of Smad7 via miR-21 adds another dimension to

BMP-Smad "canonical" pathway, and therefore, the novel regulatory mechanisms involved in maintaining/balancing signalling pathways in skin homeostasis.

Despite the recent progress in understanding the role of miR-21 in disease and its regulation by BMPs, its roles and functional significance in the skin and HF in health and disease should be investigated further.

4.3.1 MicroRNA-21 involvement in the control of gene expression in the skin during chemically induced carcinogenesis

The great majority of malignant tumours arise from the epithelial part of the skin. Despite the fact that the epidermis is one the tissues most widely used in experimental cancer research (Marks, 1976; Hennings *et al.,* 1993), our knowledge of the molecular processes that underlie epithelial proliferation and differentiation associated with the potential role of miRNAs in tumour development is still limited. Therefore, we analyzed the expression pattern of miR-21 in the skin during chemically induced carcinogenesis in FVB mice.

It was observed that expression of miR-21 increased in the hyperplastic epidermis at the earliest stages of tumour initiation, while it was very abundant in premalignant papillomas of the skin. These data are correlated with recent reports that miR-21 is an active oncogenic miRNA. It was recently reported that miR-21 is upregulated in premalignant moderately differentiated papillomas of the skin and SCC. Recently, Dziunycz and colleagues (2010) showed expression of miRNAs was altered in SCC,

and one of the miRNAs, which showed the most dramatic increases in its expression was miR-21(Dziunycz *et al.,* 2010). Furthermore, miR-21 has been shown to be overexpressed in various tumour types. MiR-21 has been implicated in the promotion of tumour growth in breast cancer (Si *et al.,* 2007), cell proliferation in pancreatic cancer (Roldo *et al.,* 2006) and exert anti-apoptotic effects in human glioblastoma cells (Du *et al.,* 2009; Qin *et al.,* 2009). Si and colleagues (2007) demonstrated that inhibition of miR-21 activity in tumour cells resulted in the regression of tumour growth (Si *et al.,* 2007). Therefore, this suggests a pro-oncogenic role for miR-21 in promoting development and maintaining SCC in part by affecting important processes such as cell differentiation, proliferation and apoptosis in the skin.

In addition, miR-21 may facilitate the promotion and maintenance of tumour development in the skin by targeting tumour suppressor genes, such as *Pten* and *Pdcd4* (Meng *et al.,* 2007; Asangani *et al.,* 2008). The importance of Pdcd4 as a tumour suppressor was shown in the skin using mouse skin during chemical induction of skin carcinogenesis. Cmarik and colleagues (1999) analysed the effects of overexpressing Pdcd4 (under K14 promoter) on tumour formation in the mouse skin. It was shown that these TG mice showed significant reduction in papilloma formation, carcinoma incidence, and papilloma-to-carcinoma conversion frequency compared with wild-type mice (Cmarik *et al.,* 1999). Furthermore, Pdcd4 was shown to inhibit neoplastic transformation in epidermal keratinocytes (Jansen *et al.,* 2005). Pten is another tumour suppressor directly targeted by miR-21. Pten was shown to

be mutated and/or underexpressed in basal cell carcinoma, SCC and melanoma of the skin, influencing cellular processes such as cell proliferation, differentiation, and programmed cell death (Endersby and Baker, 2008). Loss of Pten functions has been linked to basal cell carcinoma, through the activation of Shh (Reifenberger, 2007). Furthermore, suppression of Pten in the skin has been shown to lead to the development of SCC in humans and in mice (Ming and He, 2009). Moreover, either deletion or mutation of Pten leads to increased susceptible to tumour induction (Suzuki *et al.*, 1998). These data demonstrate that Pdcd4 and Pten have crucial roles in skin cancer development. Therefore, our data suggests that the oncogenic features of miR-21 in promoting tumour development might include the downregulation of tumour suppressors in the skin, which can lead to increased cell proliferation and apoptotic resistance.

Conclusion

MiRNAs are relatively new class of small non-coding RNAs that can regulate post-transcriptionally their mRNA targets. Since skin serves as an excellent model system for studying the biology of organ development and regeneration, delineation of the roles of miRNAs in the skin is highly important for better understanding fundamental mechanisms that govern normal development and/or which can lead to the development of skin disorders. Our study identified the role of two miRNAs in skin and HF development and cycling (miR-31 and miR-214), and a potential role for one miRNA in skin tumourigenesis (miR-21). From the data presented here the following conclusions can be made:

1. MiR-31 plays the important role in complex regulation of the gene expression programmes that control anagen progression and hair shaft formation in the HF. By targeting a number of growth regulatory molecules, transcription factors and cytoskeletal proteins, miR-31 is involved in establishing an optimal balance of gene expression in the HF required for its proper growth and hair fiber formation. Although many aspects of the miR-31-dependent effects on the HF cycling remain to be clarified, these data will help in further establishing molecular signalling networks that control organ regeneration and raise the possibility of exploring the role of miR-31 in pathobiology of distinct clinical conditions with impaired skin regeneration and hair growth.

2. MiR-214 is a novel regulator of the Wnt/β-catenin signalling pathway in keratinocytes. As a result, the importance and value of miR-214 as a regulator of Wnt/β-catenin signalling is way beyond cutaneous biology, and can be extended to other physiological processes, which have a canonical Wnt signalling pathway at its 'core'. Therefore, this makes miR-214 a potential therapeutic target in various fields such as cutaneous biology, oncology and stem cell biology. Understanding the role of miR-214 as a novel regulator that controls skin morphogenesis and regeneration will help to develop new approaches for treatment of skin disorders by targeting the activity of miR-214.

3. This study establishes a novel mechanism involved in the realisation of the BMP effects in the skin and suggests miRNAs as important regulators modulating the effects of growth factor signalling pathways on skin development and tumourigenesis. Understanding the regulatory mechanisms in skin and HF tumour development and the role of BMPs and miR-21 in skin tumourigenesis may facilitate the clinical treatment and prevention of skin and HF derived tumours.

In summary, miRNAs are potent regulators of multiple signalling pathways playing a crucial role in maintaining tissue homeostasis and pathogenesis of many diseases. The accomplishment of this project produced new information about miRNA

involvement in skin homeostasis and skin tumourigenesis. Better understanding of the roles of miRNAs will help to develop new approaches for treatment of skin disorders by targeting the activity of specific miRNAs.

Future Work

The following experimental studies will be of help to further elucidate, 1) the mechanism underlying the functions of miR-31 in the skin and HF, 2) the underlying mechanisms involved in miR-214 in the control of the canonical Wnt signalling in the skin and HF and 3) the oncogenic functions of miR-21 in the skin epithelium by:

1. Examining the role of miR-31 in human skin and HF by pharmacological modulation of miR-31 levels by using its mimics or inhibitors in organ culture
2. By targeting cytoskeletal proteins such as keratins 16 and 17, which are important in wound healing processes it will be of interest to investigate if miR-31 can regulate certain aspects of wound healing, and age-associated impaired skin repair.
3. To define the effects of the gain and loss miR-214 activity on skin development and postnatal HF growth by generating transgenic mice overexpressing miR-214 or by pharmacological modulation of miR-214 levels by using its mimics or inhibitors.
4. To identify additional targets of miR-214 in keratinocytes
5. To determine the effects of miR-21 inhibition on the distinctive stages of epidermal tumour formation during chemically induced multistage skin carcinogenesis.
6. To identify miR-21 targets in epidermal mouse keratinocytes implicated in epidermal carcinogenesis.

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APPENDIXES A

List of differentially expressed miRNAs in distinct hair cycle stages

	Anagen	Catagen	Telogen	*P*-value
mmu-let-7a	34,080	37,282	28,616	4.99E-05
mmu-let-7b	26,775	28,245	21,593	3.97E-03
mmu-let-7c	30,679	34,524	25,817	1.35E-04
mmu-let-7d	26,886	26,308	21,365	2.89E-03
mmu-let-7e	12,254	7,611	6,136	2.32E-03
mmu-let-7f	29,618	27,453	22,636	5.85E-04
mmu-let-7g	6,762	8,690	10,316	1.03E-04
mmu-let-7i	8,921	12,732	12,953	1.04E-06
mmu-miR-1	10,182	16,405	28,682	2.09E-08
mmu-miR-100	226	259	633	1.11E-16
mmu-miR-103	2,484	2,053	884	8.74E-09
mmu-miR-106a	308	322	193	1.88E-02
mmu-miR-107	2,235	1,703	793	1.40E-08
mmu-miR-10a	643	450	313	2.66E-04
mmu-miR-10b	1,871	1,807	2,449	1.29E-03
mmu-miR-125a-5p	2,408	2,538	3,562	2.22E-05
mmu-miR-125b-5p	9,991	12,985	16,895	1.02E-10
mmu-miR-126-3p	2,239	5,403	7,564	8.03E-13
mmu-miR-127	1,386	1,974	2,282	1.02E-03
mmu-miR-128a	275	287	453	1.02E-08
mmu-miR-130a	180	282	171	1.17E-06
mmu-miR-133a	1,450	2,493	4,025	1.11E-16
mmu-miR-133b	1,214	2,089	3,611	0.00E+00
mmu-miR-140*	669	341	205	2.78E-15
mmu-miR-143	941	2,705	3,587	4.02E-13
mmu-miR-145	5,257	3,950	3,341	1.18E-07
mmu-miR-146a	78	169	678	4.44E-16
mmu-miR-148a	250	854	2,796	2.48E-12
mmu-miR-150	159	215	581	1.66E-10
mmu-miR-151-5p	1,382	970	924	2.87E-06
mmu-miR-152	873	1,359	3,092	0.00E+00
mmu-miR-155	618	45	25	0.00E+00
mmu-miR-15a	875	649	989	6.91E-04
mmu-miR-15b	6,140	2,928	2,384	2.81E-09
mmu-miR-16	14,023	11,139	9,687	2.58E-02
mmu-miR-17	1,780	2,100	877	2.34E-04
mmu-miR-181a	1,253	2,207	1,267	4.99E-07
mmu-miR-182	942	666	501	1.07E-06
mmu-miR-183	1,855	935	482	2.32E-13
mmu-miR-191	3,454	3,012	1,780	2.00E-15
mmu-miR-195	4,159	3,828	6,763	0.00E+00
mmu-miR-199a-3p	2,141	6,754	14,750	0.00E+00
mmu-miR-199a-5p	207	363	641	1.55E-13
mmu-miR-19b	195	296	117	5.05E-06
mmu-miR-200a	144	842	809	4.34E-13
mmu-miR-200b	8,130	4,745	2,703	1.73E-10

mmu-miR-200c	11,342	7,148	3,421	0.00E+00
mmu-miR-203	57,573	59,271	32,593	4.44E-16
mmu-miR-205	13,774	21,404	14,430	3.63E-04
mmu-miR-20a	2,290	2,601	1,246	3.66E-04
mmu-miR-21	4,219	4,299	9,121	1.72E-06
mmu-miR-214	4,088	2,542	4,671	1.15E-04
mmu-miR-22	231	484	535	4.38E-05
mmu-miR-221	297	411	317	8.37E-06
mmu-miR-222	442	435	297	1.55E-04
mmu-miR-223	473	25	166	4.29E-09
mmu-miR-24	4,791	9,292	10,421	3.17E-07
mmu-miR-25	3,795	2,616	1,680	3.85E-14
mmu-miR-26a	17,633	26,562	26,411	1.30E-07
mmu-miR-26b	3,627	3,544	6,210	7.88E-05
mmu-miR-27a	1,744	5,366	7,887	1.13E-11
mmu-miR-27b	2,699	8,388	9,501	1.86E-12
mmu-miR-295*	5	62	4	1.37E-03
mmu-miR-29a	309	776	2,669	0.00E+00
mmu-miR-30a	445	1,226	2,074	6.16E-13
mmu-miR-30b	6,269	3,227	3,908	1.05E-04
mmu-miR-30c	7,827	4,044	3,683	1.61E-07
mmu-miR-30d	943	1,205	1,717	4.73E-12
mmu-miR-30e	95	244	514	2.11E-13
mmu-miR-31	1,136	683	68	0.00E+00
mmu-miR-320	5,704	4,424	3,131	1.93E-10
mmu-miR-322	101	115	388	5.57E-10
mmu-miR-335-5p	59	184	237	1.08E-02
mmu-miR-361	1,292	711	621	1.44E-10
mmu-miR-377	98	119	36	5.35E-05
mmu-miR-378	215	462	511	1.21E-14
mmu-miR-379	1,042	849	1,439	1.17E-10
mmu-miR-382	395	235	371	9.26E-05
mmu-miR-423-5p	688	413	345	1.77E-06
mmu-miR-429	2,107	1,808	1,089	4.88E-07
mmu-miR-434-3p	296	465	991	8.07E-13
mmu-miR-466f-3p	364	2,133	232	8.58E-04
mmu-miR-466g	79	734	49	1.01E-02
mmu-miR-467a*	31	187	20	2.28E-03
mmu-miR-467b*	47	372	22	9.44E-04
mmu-miR-486	249	294	667	5.49E-12
mmu-miR-669c	302	191	98	1.48E-04
mmu-miR-674	359	502	243	0.00E+00
mmu-miR-676	425	310	250	4.49E-09
mmu-miR-689	189	313	1,389	0.00E+00
mmu-miR-690	8,249	2,060	1,247	1.68E-08
mmu-miR-705	5,692	3,432	4,784	5.89E-05
mmu-miR-709	41,199	27,175	29,106	2.83E-11
mmu-miR-720	871	2,393	399	2.27E-11

mmu-miR-762	6,228	4,776	5,657	4.81E-02
mmu-miR-805	503	98	118	4.53E-07
mmu-miR-92a	5,013	3,545	2,200	9.70E-14
mmu-miR-92b	1,488	850	681	2.96E-05
mmu-miR-93	572	848	378	4.18E-04
mmu-miR-98	2,000	540	715	3.10E-04
mmu-miR-99a	602	624	989	9.17E-07
mmu-miR-99b	1,094	1,206	704	9.70E-06

APPENDIXES B

RIPA Buffer

Chemicals	Amount	Comapny
Tris-HCL (1M, pH 7.5)	5mls	Sigma, UK
NaCL (5M)	3mls	Sigma, UK
Triton-x100 (10%)	10mls	Sigma, UK
Doc-NA	1g	Sigma, UK
Sodium dodecyle sulphate (SDS) (10%)	1ml	Sigma, UK
Distilled Water Add up to 100mls	-	-

Transblot Buffer

Chemicals	Amount	Company
Tris Buffer	7.5g	Sigma, UK
Glycine	36g	Sigma, UK
Distilled Water Add up to 250mls	-	-

Tris Buffer (pH 8.9) (Lower stacking gel)

Chemicals	Amount	Company
Tris Base	18.16g	Sigma, UK
SDS	0.4g	Sigma, UK
Distilled Water Add up to 100mls	-	-

Tris Buffer (pH 6.9) (upper stacking gel)

Chemicals	Amount	Company
Tris Base	6.06g	Sigma, UK
SDS	0.4g	Sigma, UK
Distilled Water Add up to 100mls	-	-

Composition of electrophoresis separation gel

Chemicals	8% separation gel	3% stacking gel	Company
Bis/Acrylamide	2.0ml	375µl	Bio-Rad Corp, UK
Lower Gel Tris/*Upper gel Tris	2.5ml	*1.25ml	See above
Temed	15µl	8µl	Sigma, UK
10% Ammonium persulphate (APS)	56µl	30µl	Bio-Rad Corp, UK
Distilled water	5.5ml	3.375ml	-

Sample Buffer

Chemicals	Amount	Company
Glycerol	7mls	Invitrogen, UK
20% SDS	11.5mls	Sigma, UK
1M Tris/HCL	6.3mls	Sigma, UK
*Dithiothreitol (DTT)	1g	Invitrogen, UK
Bromophenal Blue	0.1	Bio-Rad Corp, UK

APPENDIXES C

Changes in gene expression programmers in primary mouse epidermal keratinocytes treated with sequence specific antagonist of microRNA-31

Adhesion/Extracellular Matrix	***Gene Symbol***	***Accession Number***	***Fold Change***
catenin (cadherin associated protein), alpha-like 1	Ctnnal1	NM_018761	3.28
ankyrin repeat domain 26	Ankrd26	NM_001081112	2.91
transmembrane protein 161B	Tmem161b	NM_175187	2.69
espin-like	Espnl	NM_001033292	2.54
plexin C1	Plxnc1	NM_018797	2.47
transmembrane 6 superfamily member 1	Tm6sf1	NM_145375	2.45
occludin	Ocln	NM_008756	2.43
neural cell adhesion molecule 1	Ncam1	NM_010875	2.39
FAT tumor suppressor homolog 3	Fat3	NM_001080814	2.22
junction-mediating and regulatory protein	Jmy	NM_021310	2.15
asporin	Aspn	NM_025711	2.09
cadherin	Cdh17	NM_019753	2.05
transmembrane protein 44	Tmem44	NM_172614	2.04
adhesion molecule with Ig like domain 2	Amigo2	NM_178114	2.01
CEA-related cell adhesion molecule 14	Ceacam14	NM_025957	2.00
L1 cell adhesion molecule	L1cam	NM_008478	-2.00
solute carrier family 7	Slc7a4	NM_144852	-2.00
tetraspanin 32	Tspan32	NM_020286	-2.01
solute carrier family 6	Slc6a7	NM_201353	-2.01
tenascin XB	Tnxb	NM_031176	-2.03
solute carrier family 37 , member 2	Slc37a2	NM_020258	-2.11
leucine rich repeat containing 23	Lrrc23	NM_013588	-2.13
ankyrin repeat domain 58	Ankrd58	NM_173779	-2.15
cadherin 19	Cdh19	NM_001081386	-2.22
protocadherin beta 6	Pcdhb6	NM_053131	-2.23
ankyrin repeat domain 12	Ankrd12	NM_001025572	-2.23
solute carrier family 22 (organic anion transporter), member 7	Slc22a7	NM_144856	-2.27
transmembrane protein 198	Tmem198	NM_177056	-2.28
olfactomedin 2	Olfm2	NM_173777	-2.30
leucine-rich alpha-2-glycoprotein	Lrg1	NM_029796	-2.31
transmembrane protein 132E	Tmem132e	NM_023438	-2.31
transmembrane protein 138	Tmem138	NM_028411	-2.34
solute carrier family 27	Slc27a6	NM_001081072	-2.38
claudin 18	Cldn18	NM_019815	-2.50

protocadherin beta 5	Pcdhb5	NM_053130	-2.51
fibulin 7	Fbln7	NM_024237	-2.63
solute carrier family 25, member 42	Slc25a42	NM_001007570	-2.68
solute carrier family 5 (sodium/glucose cotransporter), member 2	Slc5a2	NM_133254	-2.72
integrin, alpha E, epithelial-associated	Itgae	NM_008399	-2.77
solute carrier family 22 (organic cation transporter), member 17	Slc22a17	NM_021551	-2.82
defensin related cryptdin 20	Defcr20	NM_183268	-2.86
transmembrane 4 superfamily member 5	Tm4sf5	NM_029360	-2.88
fermitin family homolog 3	Fermt3	NM_153795	-2.96
Na+/K+ transporting ATPase interacting 3	Nkain3	NM_172987	-3.11
solute carrier family 38, member 10	Slc38a10	NM_024249	-3.12
protocadherin gamma subfamily A	Pcdhga4	NM_033587	-3.29
transmembrane and coiled-coil domains 7	Tmco7	NM_173037	-3.40
transmembrane protein 63c	Tmem63c	NM_172583	-3.43
vascular cell adhesion molecule 1	Vcam1	NM_011693	-3.43
cadherin 4	Cdh4	NM_009867	-3.55
transmembrane protein 91	Tmem91	NM_177102	-3.55
gap junction protein, gamma 2	Gjc2	NM_175452	-3.56
transmembrane protein 37	Tmem37	NM_019432	-3.62
Cell cycle/Apoptosis			
nucleolar and coiled-body phosphoprotein 1	Nolc1	NM_001039352	3.97
tubulin polymerization promoting protein	Tppp	NM_182839	3.21
Fas apoptotic inhibitory molecule 2	Faim2	NM_028224	2.96
cyclin-dependent kinase-like 2	Cdkl2	NM_177270	2.64
cylindromatosis	Cyld	NM_173369	2.45
IAP promoted placental gene	Ipp	NM_008389	2.34
Bcl2-interacting killer	Bik	NM_007546	2.23
heat shock protein 1	Hspb1	NM_013560	2.16
serine/threonine kinase 4	Stk4	NM_021420	2.05
microtubule-associated protein 1B	Mtap1b	NM_008634	2.03
cyclin-dependent kinase 7	Cdk7	NM_009874	2.00
heat shock protein family, member 7	Hspb7	NM_013868	-2.09
dedicator of cytokinesis 6	Dock6	NM_177030	-2.09
heat shock protein family, member 7	Hspb7	NM_013868	-2.20
cell death-inducing DNA fragmentation factor, alpha subunit-like effector B	Cideb	NM_009894	-2.21
B-cell leukemia/lymphoma 2 related protein A1b	Bcl2a1b	NM_007534	-2.35
cell cycle exit and neuronal differentiation 1	Cend1	NM_021316	-2.66
tumor necrosis factor receptor superfamily, member 25	Tnfrsf25	NM_033042	-2.76
Fas ligand	Fasl	NM_010177	-2.87
microtubule-associated protein 1 A	Mtap1a	NM_032393	-3.29

Cytoskeleton			
myomesin 1	Myom1	NM_010867	3.68
keratin 17	Krt17	NM_010663	3.36
keratin 14	Krt14	NM_016958	2.96
myosin, heavy polypeptide 8, skeletal muscle, perinatal	Myh8	NM_177369	2.82
myosin, light polypeptide 6, alkali, smooth muscle and non-muscle	Myl6	NM_010860	2.79
FERM domain containing 5	Frmd5	NM_172673	2.63
keratin 6A	Krt6a	NM_008476	2.58
keratin 5	Krt5	NM_027011	2.49
keratin 16	Krt16	NM_008470	2.41
cornifelin	Cnfn	NM_001081375	2.21
myosin VC	Myo5c	NM_001081322	2.01
profilin 3	Pfn3	NM_029303	-2.04
synaptopodin	Synpo	NM_177340	-2.07
spectrin beta 4	Spnb4	NM_032610	-2.09
fibrillin 2	Fbn2	NM_010181	-2.24
spectrin beta 1	Spnb1	NM_013675	-3.13
epiplakin 1	Eppk1	NM_144848	-3.15
keratin 84	Krt84	NM_008474	-4.17
fibronectin type 3 and SPRY domain-containing protein	Fsd1	NM_183178	-4.91
Metabolism			
monooxygenase, DBH-like 2	Moxd2	NM_139296	3.21
pancreatic lipase	Pnlip	NM_026925	3.07
membrane metallo endopeptidase	Mme	NM_008604	3.03
N-acylsphingosine amidohydrolase 2	Asah2	NM_018830	2.96
cubilin	Cubn	NM_001081084	2.93
establishment of cohesion 1 homolog 1	Esco1m	NM_001081222	2.89
carbonic anhydrase 8	Car8	NM_007592	2.83
iduronidase, alpha-L-	Idua	NM_008325	2.67
UDP glucuronosyltransferase 2 family, polypeptide B35	Ugt2b35	NM_172881	2.60
acylphosphatase 2	Acyp2	NM_029344	2.57
monoacylglycerol O-acyltransferase 1	Mogat1	NM_026713	2.55
X-prolyl aminopeptidase	Xpnpep3	NM_177310	2.53
oxidase assembly 1-like	Oxa1l	NM_026936	2.52
glutathione S-transferase omega 1	Gsto1	NM_010362	2.46
ubiquitin specific peptidase 33	Usp33	NM_001076676	2.44
mannoside acetylglucosaminyltransferase 3	Mgat3	NM_010795	2.38
serine racemase	Srr	NM_013761	2.23
ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 2	St8sia2	NM_009181	2.17

protein phosphatase 2, regulatory subunit B (PR 52), beta isoform	Ppp2r2b	NM_028392	2.11
cytochrome c oxidase, subunit VI a, polypeptide 1	Cox6a1	NM_007748	2.09
hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 3	Hsd3b3	NM_001012306	2.08
protein phosphatase 2, regulatory subunit A	Ppp2r1b	NM_001034085	2.08
inositol polyphosphate-5-phosphatase D	Inpp5d	NM_010566	2.07
beta galactoside alpha 2,6 sialyltransferase 1	St6gal1	NM_145933	2.03
lactamase, beta	Lactb	NM_030717	2.03
coiled-coil domain containing 47	Ccdc47	NM_026009	2.02
acyl-Coenzyme A oxidase 1, palmitoyl	Acox1	NM_015729	2.02
chondroitin sulfate synthase 3	Chsy3	NM_001081328	2.02
enolase 2, gamma neuronal	Eno2	NM_013509	-2.01
granzyme E	Gzme	NM_010373	-2.01
lysyl oxidase-like 4	Loxl4	NM_053083	-2.02
calcium/calmodulin-dependent protein kinase I gamma	Camk1g	NM_144817	-2.02
glycerol-3-phosphate dehydrogenase 1-like	Gpd1l	NM_175380	-2.03
inositol polyphosphate-4-phosphatase, type I	Inpp4a	NM_030266	-2.07
hepsin	Hpn	NM_008281	-2.08
a disintegrin and metallopeptidase domain 26A	Adam26a	NM_010085	-2.08
carboxypeptidase X 1	Cpxm1	NM_019696	-2.09
methyltransferase like 8	Mettl8	NM_145524	-2.13
peptidyl arginine deiminase, type VI	Padi6	NM_153106	-2.13
cytochrome b-245, beta polypeptide	Cybb	NM_007807	-2.16
myeloperoxidase	Mpo	NM_010824	-2.17
ATPase family, AAA domain containing 4	Atad4	NM_146026	-2.23
sphingomyelin phosphodiesterase 3	Smpd3	NM_021491	-2.24
DEAQ RNA-dependent ATPase	Dqx1	NM_033606	-2.33
esterase 22	Es22	NM_133660	-2.37
ethanolamine kinase 2	Etnk2	NM_175443	-2.50
aspartate-beta-hydroxylase	Asph	NM_133723	-2.60
carbonic anhydrase 15	Car15	NM_030558	-2.63
formiminotransferase cyclodeaminase	Ftcd	NM_080845	-2.65
cytochrome c oxidase subunit VIb polypeptide 2	Cox6b2	NM_183405	-2.70
oviductal glycoprotein 1	Ovgp1	NM_007696	-2.70
cytochrome P450, family 2, subfamily d, polypeptide 12	Cyp2d12	NM_201360	-2.72
glycosyltransferase-like domain containing 1	Gtdc1	NM_172662	-2.73
nicotinamide N-methyltransferase	Nnmt	NM_010924	-2.75

cytochrome P450, family 2, subfamily d, polypeptide 26	Cyp2d26	NM_029562	-2.91
sulfotransferase family 5A, member 1	Sult5a1	NM_020564	-3.08
hydroxysteroid (17-beta) dehydrogenase 13	Hsd17b13	NM_198030	-3.10
acetyl-Coenzyme A carboxylase beta	Acacb	NM_133904	-3.10
DIP2 disco-interacting protein 2 homolog A	Dip2a	NM_001081419	-3.13
alkaline phosphatase 3	Akp3	NM_007432	-3.17
cytochrome P450, family 2, subfamily d, polypeptide 22	Cyp2d22	NM_019823	-3.26
dual specificity phosphatase 13	Dusp13	NM_001007268	-3.40
sterol O-acyltransferase 2	Soat2	NM_146064	-3.64
fucosyltransferase 2	Fut2	NM_018876	-3.89
Molecular Channels/Transport			
solute carrier family 14 (urea transporter), member 2	Slc14a2	NM_030683	5.83
pallidin	Pldn	NM_019788	2.94
apolipoprotein L 7c	Apol7c	NM_175391	2.60
pleckstrin	Plek	NM_019549	2.57
pannexin 1	Panx1	NM_019482	2.51
sodium leak channel, non-selective	Nalcn	NM_177393	2.48
solute carrier family 2 (facilitated glucose transporter), member 13	Slc2a13	NM_001033633	2.46
SEC22 vesicle trafficking protein-like A	Sec22a	NM_133704	2.46
bicaudal D homolog 1	Bicd1	NM_009753	2.45
potassium voltage-gated channel, shaker-related subfamily, member 5	Kcna5	NM_145983	2.32
muted	Muted	NM_139063	2.25
LPS-responsive beige-like anchor	Lrba	NM_001077687	2.22
importin 11	Ipo11	NM_029665	2.18
solute carrier family 6	Slc6a12	NM_133661	2.18
transient receptor potential cation channel, subfamily M, member 3	Trpm3	NM_001035239	2.06
solute carrier family 15	Slc15a1	NM_053079	2.05
aquaporin 12	Aqp12	NM_177587	2.00
kinesin family member 9	Kif9	NM_010628	-2.00
Sec61 alpha 1 subunit	Sec61a1	NM_016906	-2.04
intersectin 1	Itsn1	NM_010587	-2.51
apolipoprotein B editing complex 1	Apobec1	NM_031159	-2.53
kinesin family member 21B	Kif21b	NM_001039472	-2.56
synaptotagmin I	Syt1	NM_009306	-3.02
cytoglobin	Cygb	NM_030206	-3.57
Proteolysis			
parkin	Park2	NM_016694	3.39
ubiquilin 3	Ubqln3	NM_198623	2.65
dipeptidylpeptidase 4	Dpp4	NM_010074	2.24

ubiquitin B	Ubb	NM_011664	2.06
lysozyme-like 1	Lyzl1	NM_026092	-2.21
meprin 1 alpha	Mep1a	NM_008585	-2.25
inter alpha-trypsin inhibitor, heavy chain 4	Itih4	NM_018746	-2.29
WAP four-disulfide core domain 15A	Wfdc15a	NM_183271	-2.61
rhomboid, veinlet-like 3	Rhbdl3	NM_139228	-2.89
protease, serine, 3	Prss3	NM_011645	-2.93
chymotrypsin C	Ctrc	NM_001033875	-2.99
serine (or cysteine) peptidase inhibitor, clade F, member 2	Serpinf2	NM_008878	-3.29
Signalling			
CD19 antigen	Cd19	NM_009844	4.36
G protein-coupled receptor 158	Gpr158	NM_001004761	3.44
sclerostin	Sost	NM_024449	3.30
CD109 antigen	Cd109	NM_153098	3.29
olfactory receptor 646	Olfr646	NM_147056	3.26
calcium/calmodulin-dependent protein kinase II inhibitor 1	Camk2n1	NM_025451	3.24
vomeronasal 1 receptor, G5	V1rg5	NM_134206	3.20
olfactory receptor 691	Olfr691	NM_147061	3.12
synaptojanin 2 binding protein	Synj2bp	NM_025292	3.01
olfactory receptor 665	Olfr665	NM_146814	2.95
olfactory receptor 1284	Olfr1284	NM_146381	2.93
CASK interacting protein 1	Caskin1	NM_027937	2.78
fibroblast growth factor 10	Fgf10	NM_008002	2.78
olfactory receptor 157	Olfr157	NM_019475	2.74
olfactory receptor 821	Olfr821	NM_146776	2.67
olfactory receptor 174	Olfr174	NM_147002	2.57
fibroblast growth factor 14	Fgf14	NM_207667	2.53
olfactory receptor 1056	Olfr1056	NM_147018	2.50
follistatin-like 5	Fstl5	NM_178673	2.47
mitogen-activated protein kinase 15	Mapk15	NM_177922	2.46
olfactory receptor 183	Olfr183	NM_146485	2.44
gephyrin	Gphn	NM_172952	2.44
angiotensin II receptor, type 1a	Agtr1a	NM_177322	2.41
BMP and activin membrane-bound inhibitor, homolog	Bambi	NM_026505	2.41
mitogen-activated protein kinase kinase kinase 2	Map3k2	NM_011946	2.39
olfactory receptor 1155	Olfr1155	NM_146643	2.38
visinin-like 1	Vsnl1	NM_012038	2.38
protein kinase C, theta	Prkcq	NM_008859	2.33
betacellulin, epidermal growth factor family member	Btc	NM_007568	2.32
glutamate receptor, metabotropic 8	Grm8	NM_008174	2.31

guanine nucleotide binding protein, alpha transducing 1	Gnat1	NM_008140	2.30
phosphatidylinositol 3-kinase, catalytic, beta polypeptide	Pik3cb	NM_029094	2.28
interleukin 1 beta	Il1b	NM_008361	2.27
olfactory receptor 552	Olfr552	NM_147102	2.24
olfactory receptor 380	Olfr380	NM_147025	2.24
olfactory receptor 273	Olfr273	NM_146824	2.19
receptor (calcitonin) activity modifying protein 2	Ramp2	NM_019444	2.18
striatin, calmodulin binding protein	Strn	NM_011500	2.17
olfactory receptor 1278	Olfr1278	NM_146394	2.16
olfactory receptor 554	Olfr554	NM_146325	2.15
olfactory receptor 615	Olfr615	NM_147080	2.14
vomeronasal 1 receptor, C33	V1rc33	NM_134436	2.11
receptor (TNFRSF)-interacting serine-threonine kinase 2	Ripk2	NM_138952	2.11
olfactory receptor 668	Olfr668	NM_147059	2.09
androgen binding protein epsilon	Abpe	NM_207262	2.09
olfactory receptor 435	Olfr435	NM_146653	2.09
signal recognition particle receptor, B subunit	Srprb	NM_009275	2.07
protein kinase C, beta 1	Prkcb1	NM_008855	2.07
hepatoma-derived growth factor, related protein 3	Hdgfrp3	NM_013886	2.07
G protein-coupled receptor kinase 4	Grk4	NM_019497	2.07
doublecortin	Dcx	NM_010025	2.06
olfactory receptor 1231	Olfr1231	NM_146454	2.05
G protein-coupled receptor 98	Gpr98	NM_054053	2.04
chemokine (C-C motif) receptor 2	Ccr2	NM_009915	2.02
progesterone receptor	Pgr	NM_008829	2.01
olfactory receptor 160	Olfr160	NM_030553	2.01
olfactory receptor 49	Olfr49	NM_010991	2.00
olfactory receptor 1100	Olfr1100	NM_146594	2.00
dapper homolog 2, antagonist of beta-catenin	Dact2	NM_172826	-2.00
ghrelin	Ghrl	NM_021488	-2.00
pleckstrin homology, Sec7 and coiled-coil domains, binding protein	Pscdbp	NM_139200	-2.03
interleukin 9 receptor	Il9r	NM_008374	-2.04
endothelin converting enzyme 2	Ece2	NM_139293	-2.04
G protein-coupled receptor 20	Gpr20	NM_173365	-2.04
adrenergic receptor, beta 1	Adrb1	NM_007419	-2.05
leukocyte-associated Ig-like receptor 1	Lair1	NM_178611	-2.05
sonic hedgehog	Shh	NM_009170	-2.06
bradykinin receptor, beta 2	Bdkrb2	NM_009747	-2.06
wingless-related MMTV integration site 11	Wnt11	NM_009519	-2.06

cholinergic receptor, nicotinic, alpha polypeptide 5	Chrna5	NM_176844	-2.06
insulin II	Ins2	NM_008387	-2.08
olfactory receptor 703	Olfr703	NM_146596	-2.08
fos-like antigen 2	Fosl2	NM_008037	-2.09
disabled homolog 2	Dab2	NM_001037905	-2.12
G protein-coupled receptor 161	Gpr161	NM_001081126	-2.12
protein kinase, cAMP dependent regulatory, type II alpha	Prkar2a	NM_008924	-2.12
platelet derived growth factor receptor, alpha polypeptide	Pdgfra	NM_011058	-2.13
interleukin 13 receptor, alpha 1	Il13ra1	NM_133990	-2.15
olfactory receptor 1036	Olfr1036	NM_207142	-2.16
ankyrin 1, erythroid (Ank1), transcript variant 2	Ank1	NM_031158	-2.16
prolactin releasing hormone receptor	Prlhr	NM_201615	-2.17
brain-specific angiogenesis inhibitor 1	Bai1	NM_174991	-2.17
prolactin family 7, subfamily b, member 1	Prl7b1	NM_029355	-2.19
bone morphogenetic protein 2	Bmp2	NM_007553	-2.19
G protein-coupled receptor 141	Gpr141	NM_181754	-2.21
olfactory receptor 476	Olfr476	NM_146924	-2.23
cholinergic receptor, nicotinic, alpha polypeptide 9	Chrna9	NM_001081104	-2.23
neurotensin receptor 1	Ntsr1	NM_018766	-2.23
calcitonin/calcitonin-related polypeptide	Calca	NM_007587	-2.23
thromboxane A2 receptor	Tbxa2r	NM_009325	-2.24
olfactory receptor 508	Olfr508	NM_146773	-2.24
interferon regulatory factor 1	Irf1	NM_008390	-2.25
prostaglandin E receptor 4	Ptger4	NM_008965	-2.26
interleukin 12 receptor, beta 1	Il12rb1	NM_008353	-2.27
inhibitor of growth family, member 3	Ing3	NM_023626	-2.27
retinoid X receptor gamma	Rxrg	NM_009107	-2.28
olfactory receptor 1245	Olfr1245	NM_146788	-2.30
glutathione peroxidase 2	Gpx2	NM_030677	-2.30
G protein-coupled bile acid receptor 1	Gpbar1	NM_174985	-2.30
olfactory receptor 720	Olfr720	NM_146392	-2.30
advanced glycosylation end product-specific receptor	Ager	NM_007425	-2.36
chemokine (C-X3-C motif) ligand 1	Cx3cl1	NM_009142	-2.36
chemokine (C-C motif) receptor-like 2	Ccrl2	NM_017466	-2.36
glutamate receptor, ionotropic, NMDA1 (zeta 1)	Grin1	NM_008169	-2.39
fibroblast growth factor receptor substrate 2	Frs2	NM_177798	-2.39
glutamate receptor, ionotropic, AMPA1 (alpha 1)	Gria1	NM_008165	-2.39

calcitonin receptor (Calcr), transcript variant a	Calcr	NM_007588	-2.42
selectin, platelet (p-selectin) ligand	Selplg	NM_009151	-2.43
olfactory receptor 360	Olfr360	NM_146622	-2.44
galanin	Gal	NM_010253	-2.44
chemokine (C-X3-C) receptor 1	Cx3cr1	NM_009987	-2.48
insulin-like growth factor 1	Igf1	NM_184052	-2.49
somatostatin receptor 1	Sstr1	NM_009216	-2.51
olfactory receptor 1352	Olfr1352	NM_147071	-2.52
G protein-coupled receptor 35	Gpr35	NM_022320	-2.53
galanin receptor 3	Galr3	NM_015738	-2.54
nephrosis 1 homolog, nephrin	Nphs1	NM_019459	-2.55
G protein-coupled receptor 84	Gpr84	NM_030720	-2.56
insulin receptor	Insr	NM_010568	-2.58
olfactory receptor 524	Olfr524	NM_001011814	-2.58
interleukin 27 receptor, alpha	Il27ra	NM_016671	-2.59
kelch-like 12	Klhl12	NM_153128	-2.61
leptin receptor	Lepr	NM_010704	-2.65
trace amine-associated receptor 9	Taar9	NM_001010831	-2.66
DAZ interacting protein 1	Dzip1	NM_025943	-2.66
cortistatin	Cort	NM_007745	-2.67
G protein-coupled receptor 77	Gpr77	NM_176912	-2.68
natriuretic peptide precursor type A	Nppa	NM_008725	-2.69
semaphorin 6C	Sema6c	NM_011351	-2.70
olfactory receptor 734	Olfr734	NM_146664	-2.73
endothelial differentiation, G-protein-coupled receptor 6	Edg6	NM_010102	-2.76
estrogen receptor 2 (beta)	Esr2	NM_207707	-2.78
olfactory receptor 1331	Olfr1331	NM_001011856	-2.78
luteinizing hormone beta	Lhb	NM_008497	-2.80
free fatty acid receptor 1	Ffar1	NM_194057	-2.81
actin-binding LIM protein 2	Ablim2	NM_177678	-2.82
gonadotropin releasing hormone 1	Gnrh1	NM_008145	-2.85
CD40 antigen	Cd40	NM_170701	-2.87
prepronociceptin	Pnoc	NM_010932	-2.88
patched homolog 2	Ptch2	NM_008958	-2.93
ficolin A	Fcna	NM_007995	-2.94
transforming growth factor, beta 1	Tgfb1	NM_011577	-3.00
interferon regulatory factor 7	Irf7	NM_016850	-3.01
olfactory receptor 1344	Olfr1344	NM_177061	-3.04
olfactory receptor 1393	Olfr1393	NM_146471	-3.05
MAGI family member, X-linked	Magix	NM_018832	-3.06
neurotrophic tyrosine kinase, receptor, type 2	Ntrk2	NM_001025074	-3.07
chordin-like 1	Chrdl1	NM_001114385	-3.15
olfactory receptor 128	Olfr128	NM_206816	-3.16
cholecystokinin B receptor	Cckbr	NM_007627	-3.17

GABA-B1a receptor	Gabbr1	AF114168	-3.24
olfactomedin 3	Olfm3	NM_153157	-3.29
interferon gamma inducible protein 47	Ifi47	NM_008330	-3.49
fibroblast growth factor 3	Fgf3	NM_008007	-3.61
glutamate receptor, ionotropic, NMDA2B	Grin2b	NM_008171	-3.74
CD22 antigen	Cd22	NM_009845	-3.75
G protein-coupled receptor 137	Gpr137	NM_207220	-3.89
olfactory receptor 609	Olfr609	NM_147082	-5.18
anti-Mullerian hormone	Amh	NM_007445	-7.41
glutamate receptor	Grin2d	NM_008172	-8.66
Transcription			
RAR-related orphan receptor gamma	Rorc	NM_011281	3.53
peroxisome proliferator activated receptor alpha	Ppara	NM_011144	3.15
pleiomorphic adenoma gene 1	Plag1	NM_019969	3.02
vestigial like 4	Vgll4	NM_177683	2.88
testis specific X-linked gene	Tsx	NM_009440	2.76
SRY-box containing gene 2	Sox2	NM_011443	2.54
peroxisome proliferator activated receptor gamma	Pparg	NM_011146	2.52
regulatory factor X, 4	Rfx4	NM_001024918	2.50
empty spiracles homolog 2	Emx2	NM_010132	2.50
activator of basal transcription 1	Abt1	NM_013924	2.48
v-myc myelocytomatosis viral oncogene homolog 1, lung carcinoma derived	Mycl1	NM_008506	2.46
jerky	Jrk	NM_008415	2.44
zinc finger protein 467	Zfp467	NM_020589	2.44
distal-less homeobox 3	Dlx3	NM_010055	2.44
T-cell leukemia, homeobox 3	Tlx3	NM_019916	2.40
zinc finger protein 9	Zfp9	NM_011763	2.30
LAG1 homolog, ceramide synthase 6	Lass6	NM_172856	2.26
NK6 transcription factor related, locus 2	Nkx6-2	NM_183248	2.22
zinc finger E-box binding homeobox 2	Zeb2	NM_015753	2.19
distal-less homeobox 1	Dlx1	NM_010053	2.14
ELK4, member of ETS oncogene family	Elk4	NM_007923	2.10
IWS1 homolog	Iws1	NM_173441	2.06
T-box 15	Tbx15	NM_009323	2.02
AF4/FMR2 family, member 4	Aff4	NM_033565	2.02
F-box and leucine-rich repeat protein 14	Fbxl14	NM_133940	-2.00
Kruppel-like factor 2	Klf2	NM_008452	-2.00
elongation factor RNA polymerase II	Ell	NM_007924	-2.02
NK2 transcription factor related, locus 6	Nkx2-6	NM_010920	-2.03
zinc finger homeobox 2 m	Zfhx2	NM_001039198	-2.09
achaete-scute complex homolog 1	Ascl1	NM_008553	-2.11
mesenchyme homeobox 1	Meox1	NM_010791	-2.12

GATA binding protein 2	Gata2	NM_008090	-2.12
SRY-box containing gene 17	Sox17	NM_011441	-2.17
forkhead box E1	Foxe1	NM_183298	-2.21
zinc finger protein 316	Zfp316	NM_017467	-2.22
sine oculis-related homeobox 1 homolog	Six1	NM_009189	-2.23
SAM pointed domain containing ets transcription factor	Spdef	NM_013891	-2.30
runt related transcription factor 3	Runx3	NM_019732	-2.36
SRY-box containing gene 1	Sox1	NM_009233	-2.39
microphthalmia-associated transcription factor	Mitf	NM_008601	-2.41
four jointed box 1	Fjx1	NM_010218	-2.48
TEA domain family member 3	Tead3	NM_011566	-2.49
snail homolog 3	Snai3	NM_013914	-2.54
SRY-box containing gene 11	Sox11	NM_009234	-2.59
aristaless related homeobox gene	Arx	NM_007492	-2.62
F-box protein 27	Fbxo27	NM_207238	-2.62
Spi-C transcription factor	Spic	NM_011461	-2.63
SRY-box containing gene 21	Sox21	NM_145464	-2.67
ring finger protein 183	Rnf183	NM_153504	-2.74
eukaryotic translation elongation factor 1 delta	Eef1d	NM_029663	-2.82
ring finger protein 186	Rnf186	NM_025786	-2.87
zinc finger protein 345	Zfp345	NM_001034900	-2.92
zinc finger protein 748	Zfp748	NM_001035231	-3.08
LIM homeobox protein 3	Lhx3	NM_001039653	-3.19
paired box gene 1	Pax1	NM_008780	-3.20
hairy and enhancer of split 5	Hes5	NM_010419	-3.31
undifferentiated embryonic cell transcription factor 1	Utf1	NM_009482	-3.96